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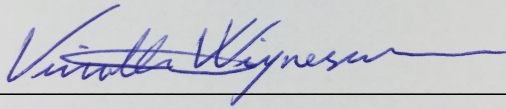
Pseudomonas putida as a microbial cell factory

PhD Thesis
by
Vinoth Wigneswaran

Department of Systems Biology
Technical University of Denmark
February 2016

Preface

This thesis is written as a partial fulfilment of the requirements to obtain a PhD degree at the Technical University of Denmark (DTU). The work was carried out from October 2010 to February 2016 at the Infection Microbiology Group, Department of Systems Biology, DTU. The study was conducted under the supervision of Associate Professor Lars Jelsbak (Department of Systems Biology, DTU), Associate Professor Anders Folkesson (National Veterinary Institute, DTU) and Professor Peter Ruhdal Jensen (National Food Institute, DTU). The work was funded by a PhD stipend from the Technical University of Denmark.

A handwritten signature in blue ink, reading 'Vinoth Wigneswaran', is centered on a light gray rectangular background. A thin horizontal line is positioned below the signature.

Vinoth Wigneswaran

Kongens Lyngby, February 2016

Summary

The extensive use of fossil fuels has a severe influence on the environment. In order to reduce the dependency on these limited resources and to protect the environment substantial effort is being made to implement renewable resources. One part of this transition is to develop methods for sustainable production of chemicals, which can be achieved by microbial cell factories.

The work presented in this PhD thesis elucidates the application of *Pseudomonas putida* as a microbial cell factory for production of the biosurfactant rhamnolipid. The rhamnolipid production was achieved by heterologous expression of the *rhlAB* operon from *Pseudomonas aeruginosa* using a synthetic promoter library in *P. putida*. Since rhamnolipid production is associated with difficulties in conventional bioreactors we have used biofilm encased *P. putida* to circumvent these problems. We show that biofilm can be used as a production platform for continuous production of rhamnolipids. A method for quantitative and qualitative analysis of the produced rhamnolipids was developed based on ultra performance liquid chromatography combined with high resolution mass spectrometry. This enabled detection of low levels of rhamnolipids.

The applicability of glycerol as a substrate was also investigated. Since glycerol is a poor substrate adaptive evolution was made in order to improve the capabilities of *P. putida* to proliferate on glycerol. The evolved lineages all had significantly increased growth rate, enhanced cell density and reduced lag phase. The genomic alterations were identified by genome sequencing and revealed parallel evolution. Glycerol was also shown to be able to support biofilm growth and as a result of this it can be used as an alternative substrate for producing biochemicals in conventional and biofilm reactors.

The use of biofilm as a production platform and the usage of glycerol as a feedstock show the potential of using microbial cell factories in the transition toward sustainable production of chemicals. Particularly, the applicability of biofilm as a production

platform can emerge as a promising alternative for producing toxic biochemicals and for producing biochemicals which are difficult to cope in conventional bioreactors.

Dansk resume

Den stigende anvendelse af fossile brændstoffer har alvorlige konsekvenser for miljøet. For at reducere afhængigheden af disse begrænsende ressourcer, og for at beskytte miljøet, bliver der gjort en stor indsats for at implementere vedvarende ressourcer. Et bidrag til denne omstilling er udviklingen af bæredygtig produktion af kemikalier, hvilket kan opnås ved brug af mikrobielle celle fabrikker.

Denne Ph.d. afhandling undersøger anvendelsen af *Pseudomonas putida* som en mikrobiel celle fabrik til fremstilling af det biologiske overfladeaktive stof (biosurfactant) rhamnolipid. Produktionen af rhamnolipider blev muliggjort ved heterologt at udtrykke *rhlAB* operonet fra *Pseudomonas aeruginosa* ved hjælp af et syntetisk promoter bibliotek i *P. putida*. Produktionen af rhamnolipider er forbundet med udfordringer i traditionelle bioreaktorer, hvorfor vi har anvendt *P. putida* biofilm for at omgå disse komplikationer. I dette studie kan vi således vise at biofilm kan benyttes som en produktions platform til kontinuerlig produktion af rhamnolipider. Kvantitative og kvalitative undersøgelser af de producerede rhamnolipider blev foretaget ved hjælp af en hertil udviklet metode, baseret på ultra højtydende væske kromatografi kombineret med høj resolution masse spektrometri. Den udviklede metode muliggjorde detektion af små mængder af rhamnolipider.

Anvendelsen af glycerol som et substrat blev også undersøgt i dette studie. Eftersom glycerol er kendt som et ringe substrat, lavede vi et adaptivt evolutions eksperiment med henblik på at optimere *P. putidas* evne til at formere sig på glycerol. De udviklede stamme udviste et signifikant øget væksthastighed, øget celle densitet samt reduceret lag fase. De bagvedliggende årsager til disse forbedringer blev undersøgt ved hjælp af genom sekventering som viste tydelige tegn på parallel evolution. Endvidere viser vi at glycerol kan benyttes til biofilm dannelse, og dermed anvendes som et alternativt substrat til fremstilling af biokemikalier i både traditionelle og biofilm reaktorer.

Både anvendelse af biofilm og brugen af glycerol viser potentialet af mikrobielle celle fabrikker til fremstilling af kemikalier på bæredygtig vis. Anvendelse af biofilm

som et produktions platform fremstår særligt som et lovende alternativ til fremstilling af giftige og komplicerede biokemikalier der er vanskelige at håndtere i konventionelle bioreaktorer.

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List of publications

Paper 1

Wigneswaran V, Nielsen K F, Jensen P R, Folkesson A, Jelsbak L (2016). Biofilm as a production platform for heterologous production of rhamnolipids by the non-pathogenic strain *Pseudomonas putida* KT2440. Submitted manuscript.

Paper 2

Wigneswaran V, Jensen P R, Folkesson A, Jelsbak L (2016). Glycerol adapted *Pseudomonas putida* with increased ability to proliferate on glycerol. Manuscript in preparation.

Paper 3

Wigneswaran V, Amador C I, Jelsbak L, Sternberg C, Jelsbak L (2016). Utilization and control of ecological interactions in polymicrobial infections and community-based microbial cell factories. Accepted F1000Research.

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Chapter 1

Introduction

During the last decades there has been an increased focus on human-induced environmental changes and their consequences. This has given rise to a clear consensus on reducing the extensive usage of fossil fuels, since this can be traced to have a severe impact on the environment (Mitigation, 2011). Additionally, the reservoirs of these resources are limited which furthermore necessitate a transition toward renewable feedstocks such as wind power, solar energy and organic material (biomass) (Scarlat et al., 2015, Ragauskas et al., 2006, Mitigation, 2011).

One way of encountering the challenges is to use microorganisms as cell factories for production of biochemicals (Ragauskas et al., 2006). Microbial cell factories offer an alternative to the fossil fuels based production toward a sustainable production of chemicals, fuels and pharmaceuticals from renewable resources (Dai and Nielsen, 2015). Although much research has been made in order to use microorganisms in sustainable production several aspect still remains to be elucidated in order to reach a fossil fuel independent society (Zargar et al., 2015). Some of the faced challenges are the complex nature of metabolism, heterologous expression of enzymes, tolerance to toxic compounds and compounds which are challenging to produce in conventional setups. Despite of this, various biochemicals have been engineered with the potential for being realised at an industrial scale production (Feldman et al., 2011, Zhu et al., 2014, Blank et al., 2012).

Several considerations needs to be evaluated in order to chose the right organism for producing a biochemical. These considerations range from substrates and production conditions to the final biochemical to be produced. The host should be able to tolerate the synthesised biochemical and the metabolic pathway for producing the compound should exist in the host or it should be possible to introduce the necessary pathways by genetic engineering. Hence, knowledge and efficient genetic tools should be avail-

able for manipulating the host (Keasling, 2010). The most widely used organism for production of biochemicals are *Bacillus subtilis*, *Escherichia coli* and *Saccharomyces cerevisiae* (Keasling, 2010).

One group of organisms with not yet explored potential is *Pseudomonas* spp. This genus has species that have many catabolic and anabolic capabilities which are of particular interest for industrial applications (Poblete-Castro et al., 2012). Furthermore these bacteria have an inherent tolerance towards many compounds (such as solvents) which makes them favourable for industrial applications. Another desirable feature is their ability to grow in the sessile mode of growth called biofilm. By growing in surface-associated biofilms the cells become more resistant towards hostile compounds in their surroundings compared to their planktonic counterparts (Li et al., 2006). Hence, these features of the biofilm mode of growth can be utilised to produce biochemicals that are detrimental for the cells at high concentrations.

In the following chapters the various aspects of producing biochemicals will be presented. Firstly an alternative substrate, crude glycerol, will be presented (Chapter 2) followed by production of a promising biosurfactant rhamnolipids (Chapter 3). This will be based on employing *Pseudomonas putida* as the host (Chapter 4) for heterologous production of rhamnolipids in a biofilm (Chapter 5). The heterologous production of rhamnolipids was achieved by employing a synthetic promoter library (Chapter 6). These are the main topics of this PhD dissertation (Chapter 7). Following the conclusion and perspectives (Chapter 8) is the research article in Chapter 10.

Chapter 2

Alternative feedstocks

The use of alternative feedstocks for production of biochemicals is of increasing interest in order to reduce the production cost and to make biobased production more cost competitive (Posada et al., 2011, Johnson and Taconi, 2007). Lignocellulosic biomass and organic by-products, such as glycerol, are particularly interesting as they are low-cost feedstocks and do not give rise to the dilemma of diverting the food supply away from the human food chain. Although the use of alternative feedstocks is attractive and the use of inexpensive waste materials from industrial processes could reduce production cost, their use is challenging owing to the presence of several microbial inhibitors which affect the subsequent processing (da Silva et al., 2009).

2.1 Crude glycerol as an alternative substrate

One example of waste material is crude glycerol derived from the biodiesel production. The use of biodiesel have increased during recent years as it can be used in conventional diesel engines with little or no major modifications (Johnson and Taconi, 2007). The challenge of using crude glycerol is the impurities present in the waste product together with the variation in composition depending on origin of production (Thompson and He, 2006, Hu et al., 2012). Although the composition and components in batches differ the predominant impurities are methanol and sodium or potassium residues from the transesterification process (da Silva et al., 2009). In addition to these compounds, heavy metals also adds on to the list of impurities (Fu et al., 2015). In addition to these impurities, crude glycerol also contains free fatty acids which together with glycerol can serve as a carbon source (Fu et al., 2014, Fu et al., 2015).

In order to use waste glycerol as substrate the employed microorganism should be able to tolerate and cope with the present impurities to achieve a sufficient production

setup. Otherwise a detoxification process may be needed which will increase the cost. One organism having a high inherent tolerance towards various inhibitory compounds is *P. putida*. This organism has been shown to be able to grow equally well in crude glycerol as in pure reagent grade glycerol (Fu et al., 2014, Fu et al., 2015). Hence, this organism is able to tolerate and circumvent the challenges there might be in using this alternative substrate without the requirement for making genetic engineering to get it to proliferate on crude glycerol.

The impurities can however in some cases be beneficial. Since crude glycerol is diluted before use the concentration of the impurities decreases. Thus, in some cases the diluted impurities can be taken advantage of by serving as nutrients. It has been shown that addition of crude glycerol alone could sustain growth and the addition of Fe and Mg salts could together with crude glycerol restore growth of *P. putida* to the level obtained when supplementing with mineral salts (Verhoef et al., 2014).

The use of crude glycerol as substrate has been investigated for production of polyhydroxyalkanoates (PHAs) used for manufacturing biodegradable plastics (Fu et al., 2014, Fu et al., 2015). Fu et al. (2015) identified changes at the transcriptomic and proteomic level when they used crude glycerol although these changes did not give rise to an alteration in growth rate. In particular genes involved in inorganic ion transport and metabolism was elevated which correlated with the known impurities present in crude glycerol.

Another example of using crude glycerol as the substrate for producing biochemicals is the production of *p*-hydroxybenzoate. Verhoef et al. (2014) investigated the effect of crude glycerol on production of *p*-hydroxybenzoate and growth of *E. coli* and *P. putida*. Surprisingly, they found a stimulating effect of crude glycerol on the performance of *P. putida* rather than hampering the cells. In contrast, *E. coli* was severely hampered by the impurities although it was able to proliferate. Hence, the choice of organism is highly important on the outcome when using crude glycerol.

Although the use of crude glycerol as a low cost feedstock is attractive when using *P. putida* as a microbial cell factory, the glycerol metabolism is not well known in *P. putida*. The use of glycerol is associated with both low growth rates and long lag

phase which is a major limitation in utilising glycerol (Nikel et al., 2014). Conducting adaptive laboratory evolution experiments could improve the glycerol utilisation as this approach has shown to be effective in improving substrate utilisation (Wisselink et al., 2007, Meijnen et al., 2008).

Chapter 3

Biosurfactant

The transition toward a sustainable green production of chemicals is a process being initiated over the last decades. There is a general agreement that a transition towards sustainable synthesis of chemicals is a necessity to preserve the environment. The development of sustainable bioprocesses for replacing the conventional petrochemical based products is however a challenging task. Although the transition toward biobased synthesis of chemicals is very appealing their commercial application is facing a major obstacle – the economy (Bozell and Petersen, 2010). However, the advances made in biotechnology combined with increased awareness among the consumers have promoted the search for more environmental friendly alternatives to the conventional petrochemical based chemicals (Banat et al., 2000).

One class of chemicals, which have gained much attention in this transition, is surfactants (Banat et al., 2000, Muller et al., 2012). Surfactants are surface active molecules able to interact between aqueous and nonaqueous interfaces. This is mediated by the amphiphilic nature of the molecules consisting of a hydrophilic head and a hydrophobic tail. This structure increases the aqueous solubility of hydrophobic liquids by reducing the surface/interface tension at the air-water and water-oil interface (Haba et al., 2003). This property makes them act as detergents, emulsifiers, foaming agent and wetting agent. Hence, the broad applicability of this group of chemicals also makes them an important and widely used class of chemicals in industry, agriculture, food, cosmetic and pharmaceutical applications (Muller et al., 2012, Henkel et al., 2012).

A substantial amount of research has been made in relation to produce surfactants by microorganisms – referred to as biosurfactants. The increased focus is supported by the fact that biosurfactants possess considerable advantages compared to their chemical counterparts. The petroleum derived surfactants are usually toxic and rarely degraded by microorganisms and therefore constitute a potential source of pollution. In

contrast, biosurfactants are more environmental friendly by being less toxic, and biodegradable. Furthermore, they have high surface activity, are more structurally diverse, and can be produced from renewable products (Haba et al., 2003, Sousa et al., 2012, Banat et al., 2010). These features also make them candidate for new applications in bioremediation of hydrocarbons from contaminated soil and water, heavy metal removal, soil washing and oil spills (Costa et al., 2010, Urum and Pekdemir, 2004, Mulligan and Wang, 2006).

Microbial surfactants are produced by many bacteria and fungi (Morita et al., 2007, Cavalero and Cooper, 2003, Moya Ramirez et al., 2015, Banat et al., 2010). A range of different biosurfactants can be produced depending on the organism and culture conditions used. The biosurfactants are classified on the basis of their chemical structure into five groups. These are glycolipids, lipopeptides, fatty acids, phospholipids, and polymeric biosurfactants (Ward, 2010). The differences in the physiochemical properties of a biosurfactant will make it more or less suitable for the application of interest.

The production of biosurfactants are not yet cost competitive in comparison to their synthetic counterparts. Hence, for biosurfactants to replace synthetic surfactants the cost of raw materials and process should be minimised. Various renewable substrates have been considered in this regard such as industrial waste. Some of these are olive oil mill effluent produced from olive oil extraction, waste cooking oil and glycerol from biodiesel production (Mercade et al., 1993, Moya Ramirez et al., 2015, Lan et al., 2015, Sousa et al., 2012). In addition to reduce the cost for substrates the use of waste materials also provide added value for these products which otherwise would be discarded and constitute an environmental disposal problem (Henkel et al., 2012).

3.1 Rhamnolipids – a promising biosurfactant

One of the biosurfactants that has gained a lot of attention is the glycolipid rhamnolipid. Rhamnolipids are a group of biosurfactants that have been known for many years. But during the last decades their potential have been acknowledged and their

wide range of applications have fuelled additional interest. Based on the recent publications and patents, rhamnolipids is the most investigated glycolipid as seen in Figure 1.

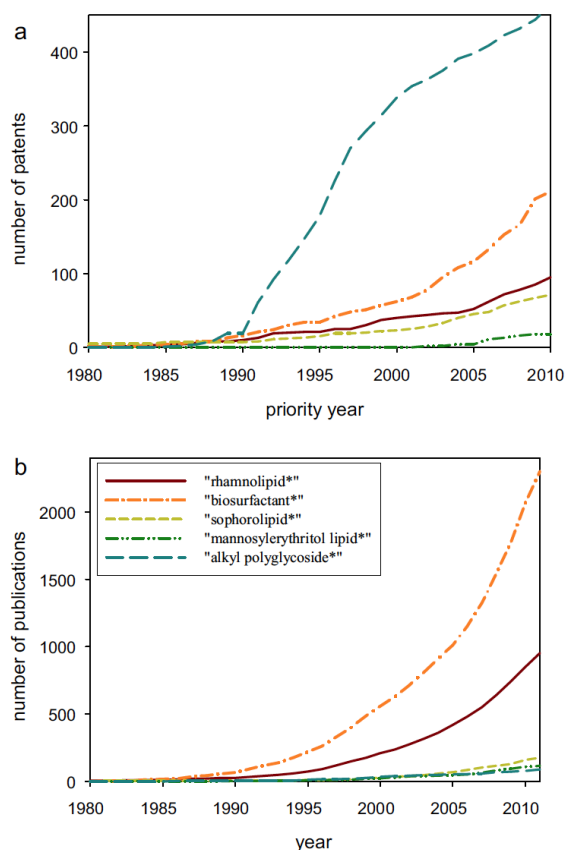


Figure 1 – The increasing interest in biosurfactants. (A) The number of worldwide patents available through European Patent Office. (B) Number of publication according to ISI Web of Science for the listed search terms (Muller et al., 2012).

Rhamnolipids were first described by Jarvis and Johnson (1949) in *Pseudomonas aeruginosa*. Similar to other surfactants, rhamnolipids are amphiphilic molecules composed of a hydrophilic part represented by rhamnose and a hydrophobic part represented by fatty acids (Figure 2). The number of rhamnose residues, fatty acid length and saturation vary depending on the employed strain and growth conditions (Deziel et al., 1999).

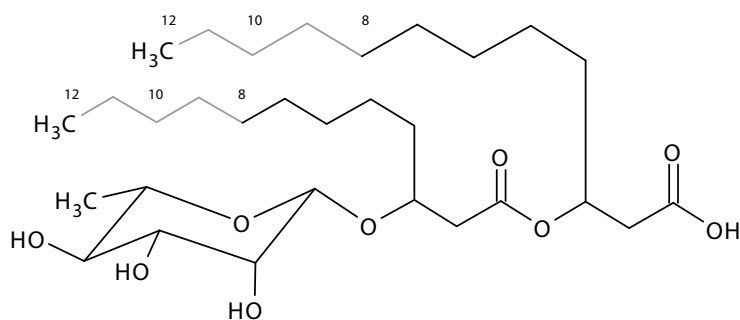


Figure 2 – Outline of the rhamnolipids. The gray part of the structure is the varying part of the rhamnolipids that differ in the different congeners. The numbers indicate the length of the fatty acid side chain.

3.2 Biosynthesis of rhamnolipids

In *P. aeruginosa* the most predominant congener is di-rhamnolipid L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (Rha-Rha-C₁₀-C₁₀) followed by the mono-rhamnolipid L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (Rha-Rha-C₁₀-C₁₀) (Deziel et al., 2000). However, other combinations of rhamnose and fatty acids are possible (Deziel et al., 1999, Rudden et al., 2015).

Three enzymes are mediating rhamnolipid synthesis in *P. aeruginosa* (Figure 3). The first step is the synthesis of rhamnose and fatty acid precursors. The lipidemic precursor is coming from *de novo* synthesis of fatty acids and the immediate precursor 3-(3-hydroxyalkanoyloxy)alkanoate (HAA) is synthesised by *rhlA* (Deziel et al., 2003, Zhu and Rock, 2008). Glucose is converted into dTDP-L-rhamnose which together with HAA are the substrates of RhlB for synthesising mono-rhamnolipids (Rahim et al., 2001). The di-rhamnolipids are synthesised by adding another rhamnose residue to the mono-rhamnolipids by *rhlC* (Rahim et al., 2001). The *rhlA* and *rhlB* genes necessary for synthesising mono-rhamnolipids constitute an operon (Pearson et al., 1997, Ochsner et al., 1994a) while *rhlC* is placed in another part of the genome (Rahim et al., 2001).

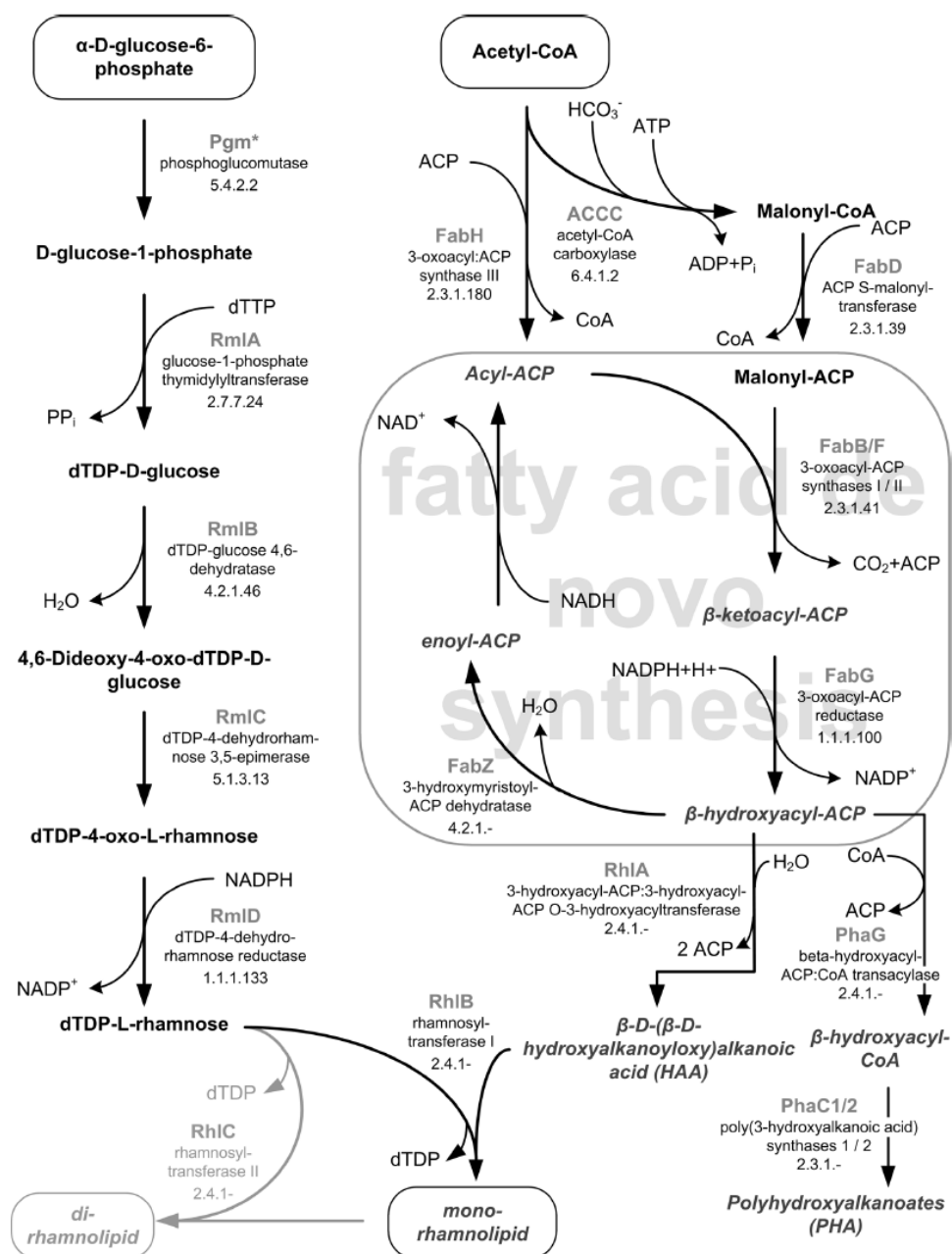


Figure 3 – Rhamnolipid biosynthesis pathway. The enzymatic steps required for producing rhamnolipids are shown. *P. aeruginosa* contains all the required genes for rhamnolipid synthesis. In *P. putida* the *rhlAB* genes need to be introduced (Wittgens et al., 2011).

The biosynthesis of rhamnolipids is highly regulated in *P. aeruginosa*. Expression of the *rhlAB* operon is under quorum sensing control and also influenced by multiple environmental factors such as availability of phosphate, nitrate, ammonium and iron (Ochsner et al., 1994b, Ochsner and Reiser, 1995, Pearson et al., 1997, Mulligan et al., 1989, Guerra-Santos et al., 1986, Mulligan and Gibbs, 1989, Deziel et al., 2003).

The pathways for the precursors exist in various bacteria as rhamnose and the fatty acids are provided from glucose and *de novo* synthesis of fatty acids respectively (Rahim et al., 2000, Aguirre-Ramirez et al., 2012, Zhu and Rock, 2008). Hence, the basis for a heterologous production of rhamnolipids exists in various organisms. Heterologous production of rhamnolipid has been achieved in recombinant *E. coli*, *P. fluorescens*, *P. oleovorans* and *P. putida* (Zhu and Rock, 2008, Ochsner et al., 1995, Wittgens et al., 2011) by introducing the *P. aeruginosa* *rhlAB* operon. A heterologous synthesis of rhamnolipids has many advantages compared to using the native host (*P. aeruginosa*). For example, it will relieve the biosynthesis from quorum sensing regulation and from the influence of external factors. This enables the possibility of engineering and modulating the biosynthesis without being obligated to consider the inherent control of the organism.

3.3 Methods for detecting rhamnolipids

Different methods can be used for detecting rhamnolipids. These methods range from simple indirect methods based on physical properties of rhamnolipids, such as alteration in interfacial tension, to more advanced methods based on mass spectrometry. In between these are colorimetric methods which are based on reactions between the rhamnose residue and a coloured chemical compound that can be quantified (Heyd et al., 2008). Examples of colorimetric methods are the anthrone and orcinol assays (Hodge, 1962, Chandrasekaran and Bemiller, 1980). These assays have been commonly used in various studies (Cha et al., 2008, Pamp and Tolker-Nielsen, 2007, Mata-Sandoval et al., 1999). The benefit of these methods is that they provide a quick and simple quantification without the need for expensive equipment. The disadvantage of these methods is the lack of discrimination of the different rhamnolipid congeners. Hence, they do not provide a precise determination of the different congeners but rather a measure of the combined quantity. Furthermore, low level of rhamnolipids cannot be detected by these assays.

For precise determination and quantification of rhamnolipid congeners mass spectrometry (MS) could be employed. Mass spectrometry is often coupled with high per-

formance liquid chromatography (HPLC) in order to separate the constituents (Deziel et al., 2000). Hence, by HPLC the sample constituents are separated followed by identification and quantification by MS. The identification of the different rhamnolipid congeners is based on their elemental composition. Structural isomers, such as Rha-C₁₀-C₈ and Rha-C₈-C₁₀, can however not be discriminated by this method. This can be made by MS/MS on the basis of the fragmentation pattern (Rudden et al., 2015). Compared to the abovementioned methods HPLC-MS provide much more information on the rhamnolipid composition and can be used to detect much lower quantities. However, this method requires advanced instrumentation and the following data analysis is more cumbersome.

The importance of discriminating the rhamnolipid congeners have been shown to be important for production of rhamnolipids as the ratio between rhamnolipid congeners can change during cultivation (Muller et al., 2010). This is of particular importance when using *P. aeruginosa* as the production host since this strain produces a range of different rhamnolipid congeners (Deziel et al., 1999, Rudden et al., 2015). Hence, for investigating such changes advance methods such as HPLC-MS are crucial, as the composition of congeners will have an effect of on the physiochemical properties of the resulting rhamnolipid mixture.

Chapter 4

Pseudomonas spp and their capabilities

Different organisms are being used as industrial workhorses. The choice highly depends on the knowledge of the organism and the methods available for genetic manipulations. *E. coli* is one of the most studied organisms, and for this reason it is one of the most important microbial cell factory. The advances made in biotechnology during the last decades have opened up possibilities for the use of alternative organisms that were not previously considered as suitable to be employed as microbial cell factories. Some of the new organisms are from the metabolically versatile genus *Pseudomonads* (Poblete-Castro et al., 2012).

Pseudomonads are Gram-negative bacteria belonging to *Proteobacteria*. They are ubiquitous due to their versatility which enable them to grow in various habitats. The members of this genus have shown to possess a remarkable inherent capacity to thrive in hostile environments and adapt to these conditions which is one of the reasons for their wide prevalence. For instance the opportunistic pathogen *P. aeruginosa* have been able to cope with the challenging conditions present in the lungs of cystic fibrosis patients (Folkesson et al., 2012). Here they are constantly challenged by the immune system as well as the extensive administration of antibiotics to eradicate their presence. Despite this, *P. aeruginosa* have managed to develop systems by which they have been able to evade the obstacles (Folkesson et al., 2012). Another example is *P. putida* which is able to thrive in highly contaminated habitats and have developed systems to degrade various toxic compounds (Faizal et al., 2005).

Although not all of these features are beneficial they exemplify some of the remarkable capabilities of this genus. Owing to these inherent abilities, these organisms have gained increasing attention in being used as microbial cell factories.

4.1 *Pseudomonas putida* – a versatile microorganism

One of the new organisms being considered as a promising industrial host is *P. putida*. It is one of the best studied species of the genus *Pseudomonas* and is a strain gaining increasing attention for its broad metabolic versatility (Poblete-Castro et al., 2012). This strain exhibits a great metabolic potential by having various chromosomally encoded pathways for catabolising a vast range of compounds (Nelson et al., 2002). Its ability to adapt to varying physiochemical conditions make them highly ubiquitous (Faizal et al., 2005).

A commonly used strain is *P. putida* KT2440 which is a TOL plasmid cured derivative of *P. putida* mt-2 (Bayley et al., 1977, Bagdasarian et al., 1981). The sequencing of the *P. putida* KT2440 genome (Nelson et al., 2002) has revealed the genomic repertoire of the organism, provided new insight into the inherent capabilities and facilitated genetic engineering of the organism. The use of *P. putida* KT2440 is interesting in an industrial setting as the strain has been approved as a biological safety strain (Federal Register, 1982).

The metabolic versatility of *P. putida* is very useful in using alternative feedstocks (Figure 4). The use of crude glycerol from the biodiesel production has for instance been shown possible without being obligated to make genetic modifications (Fu et al., 2014). Although *P. putida* possesses the ability to assimilate various substrates it cannot use pentose sugars. This would have been great as lignocellulosic biomass contains pentose sugars such as xylose and arabinose (Isikgor and Becer, 2015). However, *P. putida* can be engineered to utilise both xylose and arabinose (Meijnen et al., 2008).

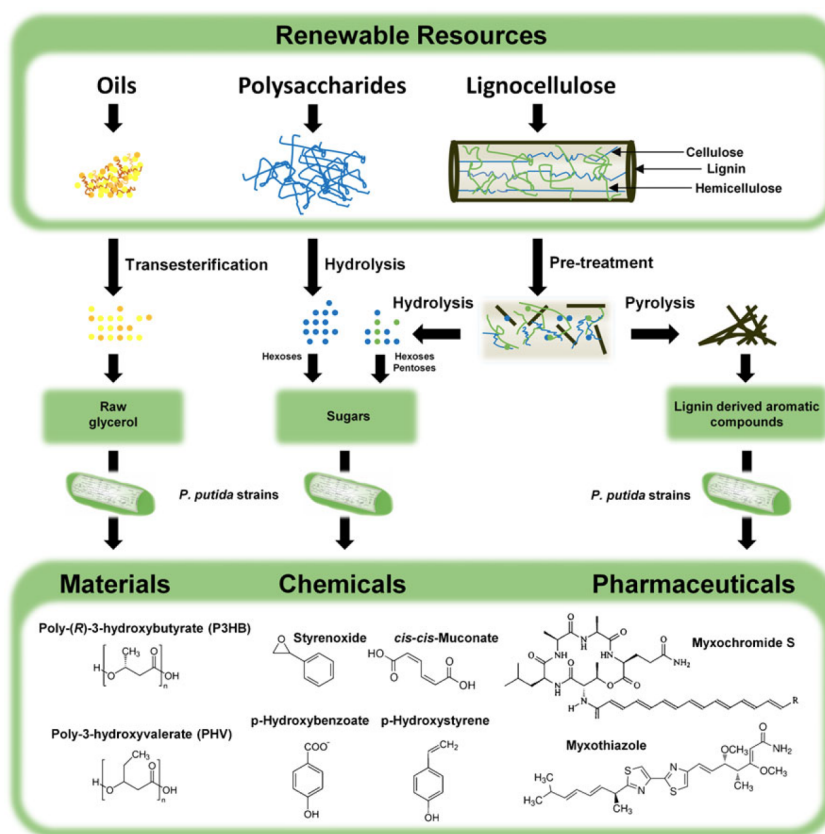


Figure 4 – The application of *P. putida* as a cell factory for biobased production of various biochemicals from renewable substrates (Poblete-Castro et al., 2012).

The inherent tolerance of *P. putida* has been shown to be very beneficial in producing biochemicals (Poblete-Castro et al., 2012, Wittgens et al., 2011). Compared to the more traditional organisms such as *E. coli*, *P. putida* has been proved to be able to cope with the impurities found in crude glycerol from the biodiesel production (Verhoef et al., 2014). This is useful as it enables the use of crude glycerol without having to detoxify the feedstock.

4.2 Industrial application of *P. putida*

The inherent capabilities of *P. putida* are not only beneficial in using alternative feed-stocks but are also very interesting for producing biochemicals (Figure 4). This was exemplified in the biosynthesis of rhamnolipids by Wittgens et al. (2011). In this study they evaluated some of the commonly used microorganisms such as *E. coli*, *B. subtilis*, *C. glutamicum* and *P. putida* for determining the best suited organism for producing rhamnolipids. The presence of high concentrations of rhamnolipids decreased the growth rate of these organisms. In general, the Gram-positive organisms were more affected than the Gram-negative organisms. In particular *P. putida* exhibited a clear advantage compared to its competitors. Hence, the inherent tolerance of *P. putida* to rhamnolipids is highly desirable as it negates the need for cumbersome genetic modifications to enable it to cope with the compound it is synthesising.

As well as its use in rhamnolipid production *P. putida* has also been useful for making the polymers polyhydroxyalkanoates (PHA) which is a large class of polyesters. Depending on the cultivation conditions, different PHA can be synthesised. Based on the knowledge of the metabolic pathways, genetic engineering can be conducted in order to redirect the production toward the compound of interest (Wang et al., 2011).

The abovementioned examples are just some of the biochemicals which can be produced by *P. putida* (Poblete-Castro et al., 2012). The advances made in genetic engineering of *P. putida* have been pivotal in enabling these modifications to be effectuated and thereby the use of *P. putida* for the production of biochemicals. The inherent tolerance of *P. putida* offers an excellent starting point for suppressing the hurdles of using and producing toxic chemical compounds of natural or heterogeneous origin. The tolerance and resistance towards chemicals can further be extended if the conventional mode of growth in planktonic cultures is replaced by the sessile mode of growth in biofilm. This mode of growth is associated with increased tolerance towards toxic compounds and has been shown to withstand much more harsh conditions than their planktonic counterparts (Li et al., 2006).

Chapter 5

Biofilm

In nature most bacteria are found in structured surface associated sessile communities called biofilms (Costerton et al., 1995). Biofilm consist of microbial cells embedded in a self-produced matrix consisting of different types of biopolymers known as extracellular polymeric substances (EPS). The EPS has multiple roles. In addition to providing structural support for the protruding biofilm, it is involved in immobilising biofilm to the surface, providing cohesiveness to the biofilm as well as enhance biofilm resistance to environmental stress (Costerton et al., 1995, Drenkard and Ausubel, 2002). Thus, the EPS provides a protective environment for the microbial cells from their surroundings.

The ubiquitous and protective nature of biofilm makes them a major challenge in various situations and make biofilm difficult to eradicate from unwanted settings – whether it is in the health care system or in industrial settings (Costerton et al., 1999). In the health care system, infections coursed by microbial biofilm are a major challenge owing to their resistance towards antimicrobial agents (Hall-Stoodley et al., 2004, Hoiby et al., 2010). In industrial settings, the presence of biofilm in pipelines and fermenters result in fluctuation in productivity and thereby hamper stable production (vellaisamy Kumarasamy and Maharaj, 2015). Similar hurdles of biofilm settling also have implications in the food industry (Winkelstroter et al., 2014). Hence, biofilms are commonly encountered in our everyday life and cause challenges in various settings.

Although the first observation of biofilm was made a long time ago (Zobell and Anderson, 1936) the observations were not followed up until several decades later (Costerton et al., 1978). The realisation of the widespread nature of biofilm, however, increased the research into elucidating the complexity of biofilm formation.

Lately, there has been an increased interest in the use of biofilms as a production platform for producing biochemicals. In this way, some of the abovementioned features have been exploited. For instance the increased tolerance towards various compounds could be taken advantage of for the production of toxic biochemicals or for the use of alternative feedstocks. Furthermore, the growth on surfaces offers other opportunities and production setups than the conventional bioreactor systems.

5.1 Considerations associated with exploiting biofilm

The increasing knowledge on biofilm biology has enabled the use of biofilm as a production platform. Currently most biological processes are made by microbial cells suspended in liquid media in batch or fed-batch cultures that are not reused but a continuous production of biochemicals in a biofilm offers interesting alternatives. They are potentially more cost effective than batch cultivation owing to reduced downtime in reactor preparation, long term activity and cleaning (Rosche et al., 2009). A continuous production for several months without the need to interrupt the process and the resistance to external interruption make them even more interesting (Li et al., 2013, Rosche et al., 2009, Gross et al., 2007). Biofilm is self-renewable, has high cell density and it has a higher intrinsic tolerance against toxic compounds than their planktonic counterpart (Gross et al., 2010, Li et al., 2006). Biofilm-based production has also been shown to be more robust in relation to contaminants. A contamination in the feedstock showed to have no effect on the biofilm reactor performance and on microbial composition (Li et al., 2013, Weuster-Botz et al., 1993). The high number of cells in the biofilm combined with continuous wash out of cells probably prevented the establishment of the contaminating organism.

The long time viability of biofilm can however also be a challenge. During long time cultivation, changes can occur in metabolic activity which can ultimately affect the production (Li et al., 2013). Furthermore, it is known that cells growing in a biofilm have a lowered metabolic activity than their planktonic counterparts (Sternberg et al., 1999). The selection of biochemicals for synthesis should therefore be carefully determined in order to make a prudent choice. This could be a biochemical whose syn-

thesis is growth independent. An example of this is rhamnolipids (Wittgens et al., 2011). The production of rhamnolipids is of particular interest as the reduced metabolic activity in a biofilm should not affect the biosynthesis and based on a metabolic network analysis, growth should be minimised in order to achieve high yields (Wittgens et al., 2011). Hence, this should result in a highly desirable combination of the parameters of interest.

The complex and dynamic nature of biofilm is a concern in utilising biofilm. During cultivation the biofilm embedded cells will be in different physiological stages as they grow and disperse as well as undergo phenotypic changes (Sternberg et al., 1999, Sauer et al., 2002, Stewart and Franklin, 2008, Werner et al., 2004, Gross et al., 2010). This can lead to genetic instability or course variations in metabolic activity resulting in fluctuations in productivity or quality (Li et al., 2013). The ability to control growth is also crucial in order to avoid clogging and the resulting downtime. Mass transfer to maintain sufficient substrate distribution as well as to remove potential toxic or inhibiting products can also be challenging (Gross et al., 2010, Gross et al., 2007).

5.2 Examples of biofilm as the production platform

To date, biofilm is primarily used in bioremediation for treatment of wastewater (Judd, 2008). The use of biofilm as a microbial biocatalyst for producing biochemicals has been exemplified in various studies exhibiting its potential (Rosche et al., 2009). The use of biofilm in producing bulk chemicals has been reported sporadically during the last decades, for example lactic acid, ethanol, and butanol (Ho et al., 1997, Demirci et al., 1997, Qureshi et al., 2004). Lately the applicability of biofilm in biosynthesis and biotransformation has also been reported for fine chemicals (Li et al., 2013, Li et al., 2006, Gross et al., 2010, Gross et al., 2007). The use of biofilm as a biocatalyst is of particular interest in producing biochemicals which are challenging in conventional cultivation setups, e.g. biosurfactants.

Different approaches have been made in order to determine a suitable design for cultivation of biofilm for producing biochemicals. This has involved various reactor designs, materials and cultivation designs for determining an optimal solution for dealing with the encountered challenges (Li et al., 2006). The choice highly depends on the organism and the synthesising compound of interest. However, the cultivation material is crucial for establishment of biofilm. The surface properties of the material determine if the bacteria are able to get established on the surface (Qureshi et al., 2005).

5.3 Methods for investigating biofilm

Different methods can be used for investigating biofilms. For visualisation of colony morphology and production of biofilm EPS, agar plates containing Congo red dye can be used (Friedman and Kolter, 2004). For fast and quantitative analysis, the crystal violet assay is commonly used (O'Toole and Kolter, 1998). In this assay, the cells are cultivated in microtiter plates and surface attached biofilm is stained and quantified by crystal violet. For following biofilm development *in situ*, a frequently used method is flow cell technology combined with confocal laser scanning microscopy (Pamp et al., 2009). By employing fluorescent reporter proteins or staining, organisms can be visualised and the biofilm development, dynamics, gene expression, imposed perturbations and spatial organisation can be investigated in real time without disrupting the biofilm. The flow system used in our laboratory is depicted in Figure 5. The medium flow through the system is aided by the pump. The bubble traps capture any air bubbles in order to avoid disruption of the biofilm. The cells are cultivated in the flow channels on glass cover slides. The system can be assembled in various setups for achieving different growth conditions as well as for withdrawing samples for further analysis. A protocol for the procedure has been described by Tolker-Nielsen and Sternberg (2011)

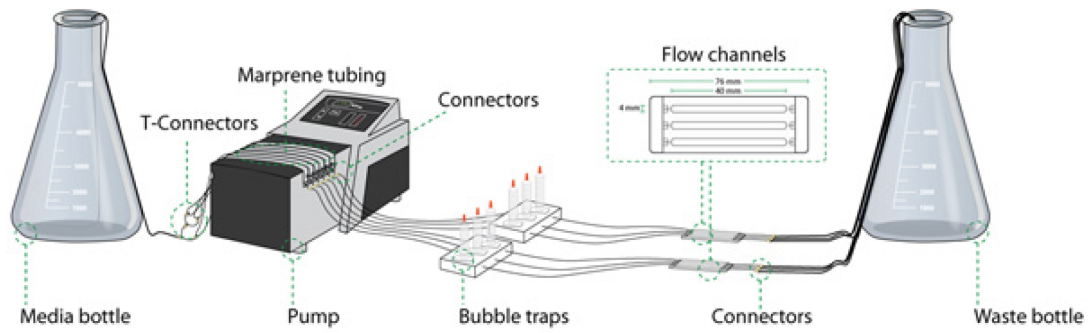


Figure 5 – Biofilm flow cell system. The media is continuously pumped through the system. The media pass through bubble traps to avoid air in the flow chambers. The cells are cultivated in the flow channels. The connectors enable various assembly possibilities for media combinations and for sample collection (Weiss Nielsen et al., 2011).

Chapter 6

Gene modulation

To have an efficient heterologous biosynthesis of a compound the necessary genes need to be expressed at a suitable level in the heterologous host. This is a necessity in order to prevent disrupting, interfering or severely hampering the host cell. Gene functionality and activity can be changed and manipulated in many ways. This can for instance be done by modulating transcription and translation levels. For example promoters (Nevoigt et al., 2006), Shine-Dalgarno sequences (Bonde et al., 2016) and transcription factors (Cox et al., 2007) can be manipulated.

Of the different possibilities for modulating gene expression, the use of promoters has been employed for many decades. (Reznikoff et al., 1969). In many cases, gene function has primarily been investigated by two extreme conditions – gene overexpression or gene inactivation. These conditions only provide limited information about the genes' effect on phenotypes or metabolic pathways. For metabolic optimisation, tuning of promoter activities is of utmost importance, as alterations may have an adverse effect on cells when using the all or nothing approach (Mijakovic et al., 2005). Furthermore, these approaches make it challenging, if not impossible to explain small effects and metabolic perturbations that will be masked by the severe disturbances the drastic changes may result in. One strategy to encounter these constraints is to find native promoters of different strengths. However, the use of an endogenous promoter has its limitations as it may be subjected to regulation. Moreover, this approach is very time consuming to carry out.

In recent years, the use of promoter libraries to make metabolic optimisation of various pathways has become widely used. These have been designed for different organisms including *E. coli* and *S. cerevisiae* (Cox et al., 2007, Nevoigt et al., 2006). Within these examples the native promoter was mutated in order to get promoters of various strengths. This enables fine-tuning of gene expression for the manipulation of pathways and for producing biochemicals.

6.1 Synthetic Promoter Library

One easy and fast approach of making promoters of various strengths is by employing the Synthetic Promoter Library (SPL) technology (Jensen and Hammer, 1998, Solem and Jensen, 2002). This method is based on the fact that some bases in a promoter sequence are more important than others when determining the strength of a promoter. Hence, by preserving some bases and randomising the surrounding bases, the promoter strength can be varied. The SPL technology can be used for creating both constitutive promoters as well as inducible promoters (Rytter et al., 2014). Constitutive promoters should be made in such a way that they provide a constant expression through the growth of the organisms. One example is the use of rRNA promoters for constitutive expression as these are some of the strongest promoters (Rud et al., 2006). Inducible promoters can also be made into promoter libraries by incorporating an operator or activator binding sites (Rytter et al., 2014). In this case, the promoters should exhibit strong control of gene expression and respond willingly to their chemical signal.

6.1.1 Designing a synthetic promoter library

Synthetic promoter libraries are based on the importance of -35 and -10 consensus sequences and the spacer region (Lodge et al., 1990, Jensen and Hammer, 1998). Jensen and Hammer (1998) showed that alteration in the -35 and -10 consensus sequences or in the spacer sequence separating these sequences results in weak promoters. Hence, the association of the consensus sequences to their surroundings is pivotal in determining the promoter activity. By alternating the adjacent bases the promoter strength can be changed.

Based on this knowledge Solem and Jensen (2002) made an easy method for making SPLs by adding the SPL to the primers used for amplifying a gene of interest (GOI). Thereby the SPL can be created by a single PCR step to obtain SPL preceding GOI. By employing a reporter gene placed downstream of the SPL the activity of the obtained promoter can be determined. Some examples of reporter genes are *lacZ* (β -galactosidase), *gusA* (β -glucuronidase), *lux* (luciferase) and *gfp* (green fluorescent protein) (Solem and Jensen, 2002, Nevoigt et al., 2006). By placing GOI between

SPL and the reporter gene the expression of GOI could be determined. This has been made in various studies in which the correlation was seen to be linear (Hansen et al., 2009, Solem and Jensen, 2002, Nevoigt et al., 2006). The SPL can also be used for an operon. In this case, the individual gene expression levels can be changes equally for both genes (Solem and Jensen, 2002).

The SPL technology has a natural selection system incorporated within, as only the strains with viable expression will proliferate. Therefore, in case the expression levels are too high these promoters will naturally be excluded from the library. If an intermediate strain, such as *E. coli*, is used in the construction it may have an effect on the SPL, as the intermediate strain may not be able to cope with the expressed GOI, for instance if the product is toxic to *E. coli* but not for the intended host. Nonetheless a strong overexpression of a non-toxic protein can also be lethal. In *E. coli*, competition for the ribosomes reflected in a decrease in growth rate and ultimately resulted in cell death (Dong et al., 1995).

The SPL technology can be used for many different organisms (Solem and Jensen, 2002). The same promoter library can however not necessarily be used in different organisms. Strong promoters made in the Gram-positive bacterium *L. lactis* did not result in high expression in the Gram-negative bacterium *E. coli*, although the promoter was functional (Jensen and Hammer, 1998). This is likely to be owed to differences in recognition and preference of promoters in these organisms. However, in some cases, the differences in promoter activity is limited as has been shown for *P. aeruginosa* and *E. coli* (Lodge et al., 1990). This could be a consequence of both organisms being Gram-negative.

6.1.2 Construction of a synthetic promoter library

When constructing a SPL of strong promoters, the library can be based on rRNA promoters (Rud et al., 2006). Since promoters can be organism-specific they should preferably be based on functional promoters from the organism of interest. Following an alignment of the determined promoters the consensus sequence can be found and the -35 and -10 consensus sequence determined (Figure 6A). In this example the

promoters from *P. putida* and *P. aeruginosa* were employed for determining a SPL sequence which can be used in both organisms. Depending on the desired variability in SPL, the consensus sequences can be preserved or be doped. The surrounding bases should be randomised to provide variation of promoter strength (Figure 6B). The sequence stated in Figure 6B constitutes the SPL and can be added to primers used for amplification of GIO.

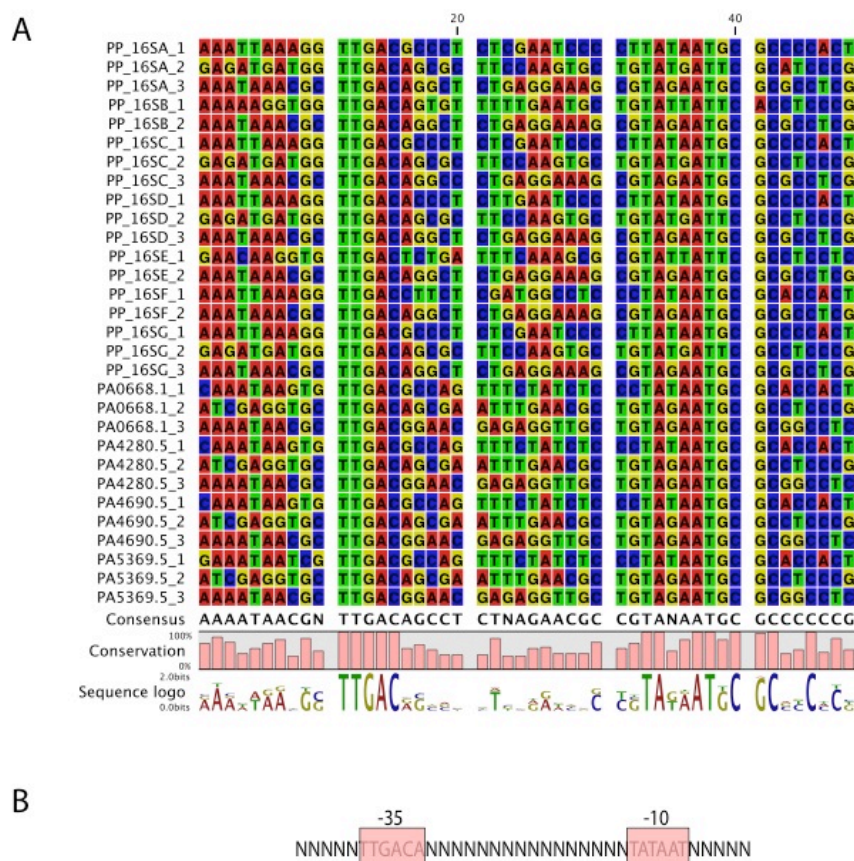


Figure 6 – Design of a synthetic promoter library. (A) The rRNA promoter sequences from *P. putida* and *P. aeruginosa* have been aligned and the consensus sequence determined. Based on the alignment, a promoter sequence can be made as illustrated in (B). The -35 and -10 consensus sequences were preserved and the surrounding bases randomised for modulating the promoter strength. The consensus sequences can however also be randomised or doped in order to increase the variability of promoter strengths in the library. The consensus sequences are highlighted in pink boxes.

Chapter 7

Present investigation

7.1 Rhamnolipid production using biofilm as the production platform

To produce rhamnolipids at an industrial scale, it is not optimal to use an opportunistic pathogen, which would be the case if *P. aeruginosa* is used. Hence, if the biosynthetic pathway could be moved to another safe organism the concerns about pathogenicity can be evaded. However, several considerations need to be made in order to choose the right organism. The organism should be able to produce rhamnolipids at high concentrations, either natively or be engineered to do so, and be able to tolerate high concentrations of rhamnolipids. In case the pathway is transferred for heterologous production, the growth phase dependent production and quorum sensing regulation should be relieved when possible.

In this PhD study, we wanted to make a proof of concept of using biofilm encased *P. putida* as a production platform for producing rhamnolipids (**Paper 1**). Rhamnolipids were chosen as the compound of interest since they have been shown as a promising alternative to the conventional surfactants (cf. chapter 3.1). Furthermore, since rhamnolipids are biosurfactants they incur major challenges during production via the conventional fermentation setup owing to foam production (Muller et al., 2010, Reiling et al., 1986, Urum and Pekdemir, 2004). Our hypothesis is that by using biofilm as a production platform these obstacles can be counteracted. Furthermore, since rhamnolipids have shown to hamper growth they also serve as a model compound for producing toxic biochemicals. The use of both *P. putida* and biofilm offer a unique combination of a production host possessing an immeasurable inherent metabolic capability and the ability to tolerate various compounds combined with the use of biofilm which is known for its high robustness towards external challenges (cf. chapter 4 and 5).

Several surfactants have been shown to interfere and disrupt biofilm formation of various organisms. The surfactant can act on the biofilm by several means, such as antimicrobial agents, by disrupting biofilm and by degrading matrix components. The biosurfactants sophorolipids and rhamnolipids have shown to exhibit antimicrobial activity against both Gram-positive and Gram-negative bacteria (Diaz De Rienzo et al., 2016a). They can also act as an anti-adhesive agent and exhibit biofilm disruptive abilities resulting in an earlier onset of biofilm detachment (Boles et al., 2005, Irie et al., 2005, Diaz De Rienzo et al., 2016b). This is likely to be effectuated by degrading the components of the biofilm matrix as rhamnolipids can cause release of lipopolysaccharides (Al-Tahhan et al., 2000). The biofilm matrix may then be destabilised by high levels of rhamnolipids. In *P. aeruginosa* the rhamnolipid production has been shown to be involved in structural organisation of the cells. This was mediated by cell motility as rhamnolipid deficient cells had changed biofilm morphology (Pamp and Tolker-Nielsen, 2007). Based on these studies we suspected that a heterologous expression of rhamnolipids would have an effect on the biofilm capabilities of *P. putida*. To clarify this and to investigate the alterations the biosynthesis of rhamnolipids can have on a heterologous host, we developed a Synthetic Promoter Library, which could reveal the implications of producing rhamnolipids and for obtaining an expression which is not influenced by any inherent regulatory systems (cf. chapter 6.1).

For detection of the produced rhamnolipids, we developed a method based on ultra performance liquid chromatography combined with high resolution mass spectrometry. This method enabled detection of small quantities of rhamnolipids, as a continuous production in a biofilm will result in lower titers than in batch cultivation. The method also enables the detection and characterisation of the different rhamnolipid congeners produced by the engineered strains (cf. chapter 3.3).

The employment of biofilm as the production platform for rhamnolipids in **Paper 1** is not limited to this compound. It only exemplifies the potential of the system and provides an alternative to produce biochemicals which can be troublesome to produce in conventional fermentations.

7.2 Alternative feedstock and future production scenarios

In order to produce cost-competitive biochemicals, it is necessary to reduce the production cost as much as possible. In the quest for reducing expenses we have investigated the use of glycerol as an alternative substrate in **Paper 2** (cf. chapter 2). Since glycerol is a poor substrate of *P. putida* an adaptive laboratory evolution experiment was made in order to improve growth rate. Furthermore, the usability of glycerol for supporting biofilm growth was investigated as most studies of *P. putida* are made using citrate as the carbon source.

Lastly, some future applications and thoughts on production setups have been presented in **Paper 3**. The employment of various species into consortia for producing biochemicals is a field gaining increasing attention. The increasing knowledge of new species allows alternative production setup in which multiple organisms can be employed to utilise and produce compounds that are not achievable by using single organisms. In **Paper 3** we present thoughts on how some of the recent studies exhibit evidence of how to construct synthetic communities. In this way artificial systems can be created composing of different consortia for bioconversion of successive steps of both substrates and intermediates for complex product formation. This could be used to develop a building block system in which different organisms can be combined based on the available substrate and product to be synthesised. In this way, it may not be necessary to transfer complex pathways between organisms. This is of particular interest when new organisms are found and their genomic potential revealed by genome sequencing. However, this is a future scenario.

Chapter 8

Conclusions and perspectives

The results from this PhD study show that it is possible to use surface-associated biofilm as a production platform for producing rhamnolipids. The challenges there may be in producing biochemicals that could potentially disrupt biofilm formation were not evident in this study. On the contrary, rhamnolipid production was in fact seen to stimulate biofilm production by facilitating cell motility. Hence, it offers an alternative to conventional setups and avoids some of their limitations.

The engineered strains for producing rhamnolipids in this study have not been subjected to any modifications for optimising the production. This could be achieved by removing the polyhydroxyalkanoate formation, as it is a competing pathway for the precursor β -hydroxyacyl-ACP used by the RhlA enzyme. By deleting the *phaCI* gene, approximately seven times more rhamnolipids could be produced (Wittgens et al., 2011). Another possibility for increasing rhamnolipid production could be by enhancing biofilm biomass. Mutating the *lapG* gene will result in an increased biofilm formation as the dispersal is hampered (Gjermansen et al., 2010, Yousef-Coronado et al., 2011). By having more cells encapsulated in the biofilm, more rhamnolipids could be synthesised. These aspects need to be addressed in order to increase rhamnolipid production in the biofilm.

Furthermore, a scale up of the rhamnolipid production is necessary in order to investigate the real life applicability. The employed setup in this study is difficult to scale as it was intended as a proof of concept. The use of *P. putida* in large-scale biofilm reactors was however attempted during this PhD study. This was done in both an annular biofilm reactor (BioSurface Technologies Corporation, Bozeman, USA) and by employing the BioBooster reactor (Grundfos, Denmark) (Lou et al., 2014). Particularly the BioBooster reactor offered some interesting features, such as the capacity to maintain a uniform biofilm thickness by growing them on closely packed dishes. Unfortunately, we encountered some challenges in establishing biofilm on the plastic

surfaces used in both the annular biofilm reactor and the BioBooster. *P. putida* was not able to form biofilm on these surfaces. It may be possible to evade this limitation by employing high biofilm producing strains, for instance a *lapG* mutant of *P. putida*. However, this was not possible at that moment due to the classification of the pilot plant. In future studies, it will be relevant to investigate this setup for a large-scale production. Alternatively, some of the other available biofilm reactors could be considered, such as the trickle-bed biofilm reactor. The upscaling of the rhamnolipid production from our proof of concept study is critical for evaluating the applicability in a viable industrial production.

A combination of using glycerol for producing rhamnolipids would be a natural continuation of the projects (Paper 1 and 2), as we showed glycerol could support biofilm growth. We have evolved strains with an increased growth rate and identified the mutations giving rise to this enhancement. Further studies are, however, necessary in order to be able to unravel the precise molecular mechanisms of these mutations. Based on the gained knowledge of glycerol metabolism from the evolved strains, new strains could be engineered which have an enhanced utilisation of glycerol and are able to produce rhamnolipids. In this process the aforementioned considerations for improving rhamnolipid synthesis should be included. Thus, an optimised rhamnolipid producing strain can be engineered using glycerol as the substrate.

Other alternative feedstocks may also be considered, for instance lignocellulosic biomass. The challenges of using such feedstocks are the presence of microbial inhibitors, such as furfural (Behera et al., 2014). We did some preliminary studies elucidating the tolerance of *P. putida* to furfural and its effect on biofilm formation. These results indicated high tolerance of *P. putida* toward furfural both as planktonic cells and in biofilm. Hence, this could be a relevant alternative to pursue.

One of the major obstacles in using biofilm as the production platform is the difficulty of controlling the system. For biofilm to be an alternative for conventional fermentation setups more knowledge should be gained in understanding and ideally controlling development of biofilm. The inherent heterogeneity of biofilm combined with the lowered metabolic activity makes production of biochemicals troublesome in terms of fluctuations as well as low production in case it is growth dependent. How-

ever, biofilm offers many features that are invaluable in a production setup, such as high resistance and robustness. Hence, once the obstacles of heterogeneity have been overcome, biofilm will be a favourable alternative in producing biochemicals that are difficult in conventional bioreactors, as exemplified with rhamnolipids, and for producing toxic compounds.

The work presented in this PhD study was based on a single specie organism. The use of multi species communities as a production platform is another aspect which should be considered in future studies. Multi species consortia are the most prevalent form of life in nature and are of utmost interest for being employed in biotechnological applications. By employing multiple species, the community could exhibit features which are otherwise impossible to achieve. The use of multiple species is however challenging, as it is very complicated to handle and control multiple organisms simultaneously. Recent studies have however, highlighted the possible application and construction of artificial interdependence of organisms making such communities possible (Zhou et al., 2015, Minty et al., 2013). Similar studies could be made for combining a rhamnolipid producing strain with a strain converting xylose into other substrates. Xylose degradation can be engineered in *E. coli* (Zhang et al., 2015). By combining these two organisms, the *E. coli* strain can provide the substrate for *P. putida*, and the *P. putida* can produce the rhamnolipids. Since *P. putida* grow very poorly on xylose (Meijnen et al., 2008) and the *E. coli* growth is hampered by rhamnolipids at high concentrations (Wittgens et al., 2011), a mutual interdependence will exist between the organisms preventing them from outcompeting each other. The mutual ratio between these organisms will in this case be pivotal. An alternative option could be a community based on the fungus *Trichoderma reesei* and *P. putida*. In this consortium the fungus can degrade lignocellulose into monomers (Minty et al., 2013) which subsequently can be used by *P. putida*. These are some examples of how this study can be extended to use microbial consortia for producing rhamnolipids by alternative substrates. As a result, this field of research assure to be very promising and has the potential to expand the possibilities within biotechnology considerably.

Chapter 9

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Chapter 10

Research articles

The research articles are enclosed in the following order.

Paper 1

Wigneswaran V, Nielsen K F, Jensen P R, Folkesson A, Jelsbak L (2016). Biofilm as a production platform for heterologous production of rhamnolipids by the non-pathogenic strain *Pseudomonas putida* KT2440. Submitted manuscript.

Paper 2

Wigneswaran V, Jensen P R, Folkesson A, Jelsbak L (2016). Glycerol adapted *Pseudomonas putida* with increased ability to proliferate on glycerol. Manuscript in preparation.

Paper 3

Wigneswaran V, Amador C I, Jelsbak L, Sternberg C, Jelsbak L (2016). Utilization and control of ecological interactions in polymicrobial infections and community-based microbial cell factories. Accepted F1000Research.

Paper 1

Biofilm as a production platform for heterologous production of rhamnolipids by the non-pathogenic strain *Pseudomonas putida* KT2440

Wigneswaran V, Nielsen K F, Jensen P R, Folkesson A, Jelsbak L (2016)
Submitted manuscript.

1 **Biofilm as a production platform for heterologous production**
2 **of rhamnolipids by the non-pathogenic strain *Pseudomonas***
3 ***putida* KT2440**

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17 Keywords: Rhamnolipids, biosurfactants, heterologous biosynthesis, *Pseudomonas*
18 *putida* KT2440, biofilm, UHPLC-HRMS, UHPLC-MS/HRMS

Abstract

Background: Although a transition toward a sustainable production of chemicals is needed, some biochemicals like biosurfactants are troublesome to produce by conventional bioreactor setups. Alternative production platforms such as surface-attached biofilm populations could potentially overcome these problems. Rhamnolipids are a group of biosurfactants highly relevant for industrial applications. Unfortunately, they are mainly produced by the opportunistic pathogen *Pseudomonas aeruginosa* using hydrophobic substrates such as plant oils. As the biosynthesis is tightly regulated in *P. aeruginosa* a heterologous production of rhamnolipids in a safe organism can relieve the production from many of these limitations and alternative substrates could be used.

Results: In the present study a heterologous production of biosurfactants was investigated using rhamnolipids as the model compound in biofilm encased *Pseudomonas putida* KT2440. The *rhlAB* operon from *P. aeruginosa* was introduced into *P. putida* to produce mono-rhamnolipids. Designing and employing a synthetic promoter library relieved the regulation of rhamnolipid synthesis and provided varying expression levels of the *rhlAB* operon resulting in different levels of rhamnolipid production. The biosynthesis of rhamnolipids decreased growth rate and stimulated biofilm formation by enhancing cell motility. Continuous rhamnolipid production in a biofilm was achieved using flow cell technology. Quantitative and structural investigations of the produced rhamnolipids were made by ultra performance liquid chromatography (UHPLC) combined with high resolution mass spectrometry (HRMS) and tandem HRMS. The predominant rhamnolipid congener produced by the heterologous *P. putida* biofilm was mono-rhamnolipid with two C₁₀ fatty acids.

Conclusion: This study shows a successful application of synthetic promoter library in *P. putida* KT2440 and a heterologous biosynthesis of rhamnolipids in biofilm encased cells without hampering biofilm capabilities. These findings expands the possibilities of cultivation setups and paves the way for employing biofilm flow systems as production platforms for biochemicals which, as a consequence of physiochemical properties are troublesome to produce in conventional fermenter setups, or for production of compounds which are inhibitory or toxic to the production organisms.

Introduction

Rhamnolipids is a group of biosurfactants with great industrial potential. Their low toxicity and biodegradability combined with their potent surface tension reducing and emulsifying activity has made them one of the most studied biosurfactants. Their possible applications range from industry, agriculture and bioremediation to personal care and medicine (Muller et al., 2012, Henkel et al., 2012). Rhamnolipids were first described by (Jarvis and Johnson, 1949) and are produced by various organisms but mainly known from the opportunistic pathogen *Pseudomonas aeruginosa* in which most studies have been made.

The rhamnolipids encompass a diverse group of compounds composed of one or two rhamnose molecules linked to one or two β -hydroxy fatty acids by β -glycosidic bonds. The rhamnose and fatty acid composition depend on strain and growth conditions (Deziel et al., 1999, Muller et al., 2011). The most abundant rhamnolipid congener produced by *P. aeruginosa* is the di-rhamnolipid L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (Rha-Rha-C₁₀-C₁₀) (Deziel et al., 1999).

66 The rhamnose moiety in rhamnolipids is synthesised from glucose (Rahim et al.,
 67 2000, Aguirre-Ramirez et al., 2012) and the fatty acid is provided from *de novo* syn-
 68 thesis (Zhu and Rock, 2008). Three enzymes mediate the synthesis of rhamnolipids in
 69 *P. aeruginosa*. The first step is the synthesis of 3-(3-hydroxyalkanoyloxy)alkanoate
 70 (HAA) mediated by RhIA (Deziel et al., 2003, Zhu and Rock, 2008). HAA is the li-
 71 pidic precursor that together with dTDP-L-rhamnose is the substrate of RhIB for syn-
 72 thesising mono-rhamnolipids (Rahim et al., 2001). The rhamnosyltransferase II (*rhlC*)
 73 is responsible for addition of another rhamnose moiety to make di-rhamnolipid
 74 (Rahim et al., 2001). The *rhlA* and *rhlB* genes constitute an operon (Pearson et al.,
 75 1997, Ochsner et al., 1994) while *rhlC* is placed in another part of the genome (Rahim
 76 et al., 2001). Expression of the *rhlAB* operon in *P. aeruginosa* is highly regulated at
 77 multiple levels and subjected to both quorum sensing control as well as regulation by
 78 environmental factors such as phosphate, nitrate, ammonium and iron availability
 79 (Ochsner and Reiser, 1995, Pearson et al., 1997, Mulligan et al., 1989, Guerra-Santos
 80 et al., 1986, Mulligan and Gibbs, 1989, Deziel et al., 2003).
 81 The complex regulation of rhamnolipid synthesis makes it difficult to control biopro-
 82 duction. Furthermore, *P. aeruginosa* is an opportunistic pathogen. To overcome these
 83 limitations various studies have been made in order to produce rhamnolipids in a het-
 84 erologous host such as *Escherichia coli*, *Pseudomonas fluorescens*, *Pseudomonas*
 85 *oleovorans* and *Pseudomonas putida* (Zhu and Rock, 2008, Ochsner et al., 1995,
 86 Wittgens et al., 2011) by introducing the *rhlAB* operon. The ability of the host to tol-
 87 erate rhamnolipid production can be a challenge as these molecules hamper growth at
 88 high concentrations (Wittgens et al., 2011). However, by using a species from the
 89 same genus a high inherent tolerance can be achieved as shown for *P. putida*
 90 (Wittgens et al., 2011, Ochsner et al., 1995).

91 The heterologous rhamnolipid production in *P. putida* KT2440 has so far been exam-
92 ined in conventional bioreactor systems with planktonic cells (Wittgens et al., 2011,
93 Ochsner et al., 1995). This type of growth is associated with difficulties owing to
94 foam formation caused by aeration of the culture (Muller et al., 2010, Reiling et al.,
95 1986, Urum and Pekdemir, 2004). We hypothesize that cultivation of cells in a sur-
96 face attached biofilm can reduce this problem. In contrast to planktonic growth, bio-
97 films represent a sessile mode of growth, and cells growing in a biofilm have a low-
98 ered growth activity compared to their planktonic counterparts (Sternberg et al.,
99 1999). In rhamnolipid production this phenotype is desirable as production is growth
100 independent and growth should be minimised in order to achieve high rhamnolipid
101 yields (Muller et al., 2010, Wittgens et al., 2011).

102 In the present study we use *P. putida* KT2440 biofilm as a production platform for
103 heterologous production of rhamnolipids by constructing a synthetic promoter library
104 (SPL). We show that producing rhamnolipids in a biofilm eliminates the formation of
105 foam, which in other production setups result in significant challenges. A synthetic
106 promoter library was designed and constructed to obtain various expression levels of
107 rhamnolipid synthesis and to evaluate the effect of biosurfactant production on cells
108 and on biofilm capabilities. A method for characterisation and quantification of the
109 produced rhamnolipids was developed based on ultra-high performance liquid chro-
110 matography (UHPLC) combined with high resolution (HRMS) and tandem mass
111 spectrometry (MS/HRMS).

113 **Results**

Using a synthetic promoter library to modify expression of *rhlAB* in *P. putida*

A previous study reported that the *rhlI* and *rhlR* need to be present in case the native *rhlAB* promoter from *P. aeruginosa* should be employed in a heterologous host (Ochsner and Reiser, 1995). Thus, for achieving high expression levels and to evaluate the influence of rhamnolipid production on cells and biofilm formation, strains capable of producing rhamnolipids at different levels were engineered by constructing a synthetic promoter library (SPL) (Solem and Jensen, 2002) to drive expression of the *rhlAB* biosynthesis genes.

The synthetic promoter library was constructed based on 16S rRNA promoter sequences from *P. putida* KT2440 and *P. aeruginosa* PAO1 in order to achieve strong constitutive promoters (Rud et al., 2006). The SPL was designed by preserving the consensus sequences and degenerating the surrounding bases to modulate promoter strength as previously described (Jensen and Hammer, 1998, Solem and Jensen, 2002). The SPL was placed in front of the *rhlAB* operon to achieve varying expression levels of the rhamnolipid biosynthesis genes (Figure 1A). A *gfp* reporter was placed downstream of the *rhlAB* operon for determining the promoter strength. The promoter activities were determined at the single cell level by monitoring the *gfp* expression. This enabled indirect monitoring of the rhamnolipid biosynthesis.

The wild type strain and a strain harbouring the *gfp* reporter vector without a promoter showed no significant difference in fluorescence intensity (Figure 1B). The promoter activity of the *rhlAB* operon without SPL and the native promoter from PAO1 showed a small but significant increase in activity compared to the reference vector, indicating an inherent but low promoter activity ascribed to the *rhlAB* operon. However, in these two cases rhamnolipid production could not be detected (data not shown). The constructed SPL resulted in a diverse set of synthetic promoters with

140 varying strength (Figure 1C). The majority of the synthetic promoters supported high-
141 er *gfp* expression levels than the control strains. An investigation of the SPL activity
142 revealed a distribution of expression levels with most promoters between 10 and 50
143 a.u.

145 **Gfp expression correlates with rhamnolipid production**

146 The synthetic promoter strength determined by Gfp fluorescence should reflect a cor-
147 responding expression of the *rhlAB* operon (Figure 1A). To evaluate the correlation
148 between fluorescence intensity and *rhlAB* expression we analysed rhamnolipid pro-
149 duction in a selection of strains with synthetic promoters of different strengths. To
150 this end, we developed a method based on ultra performance liquid chromatography
151 (UHPLC) combined with high resolution mass spectrometry (HRMS) and tandem
152 HRMS for simultaneous quantitative and qualitative analysis of the produced rhamno-
153 lipids (Supporting information Figure S1 and Table S1). The rhamnolipid congeners
154 were identified and quantified based on their elemental composition (Figure 2) and
155 were verified by positive and negative electrospray ionisation in UHPLC-HRMS.
156 Although a mixture of rhamnolipids could be produced by *P. putida*, the predominant
157 congener was Rha-C₁₀-C₁₀ followed by Rha-C₁₀-C₁₂, Rha-C₁₀-C_{12:1} and Rha-C₈-C₁₀
158 (Supporting information Figure S1). The structural composition was elucidated by
159 MS/HRMS fragmentation pattern (Supporting information Table S1). These results
160 are in accordance with previous results (Deziel et al., 1999, Rudden et al., 2015). The
161 elution pattern supported the determined structural composition.
162 Analysis of rhamnolipid production in a selection of 6 strains with different synthetic
163 promoter strengths revealed a clear linear correlation between Gfp activity and Rha-

C₁₀-C₁₀ concentration (Figure 3). Hence, Gfp fluorescence can be used as an indirect measure of rhamnolipid production.

The cost of producing rhamnolipids

The wide range of synthetic promoter strengths made it possible to investigate the metabolic load associated with rhamnolipid production. We found that increased rhamnolipid production is correlated to a reduction in growth rate (Figure 4). A 10 fold increase in rhamnolipid production result in a 14% lower growth rate.

Heterologous expression of rhamnolipids in *P. putida* result in enhanced biofilm production

Rhamnolipids are biosurfactants that may potentially interfere with *P. putida* biofilm formation (Diaz De Rienzo et al., 2016). To investigate the effect of rhamnolipid production on biofilm capabilities of the engineered strains, we used microtiter plate based assays to measure biofilm formation as a function of time. In these assays, the biofilm development of all strains followed a similar biofilm developmental progress with dispersal of the biofilm upon reaching their maximal quantity (Figure 5) as previously described for *P. putida* biofilm formation (Gjermansen et al., 2010). Surprisingly, the rhamnolipid producing strains had an enhanced biofilm production and reached a higher biomass compared to the reference strain. The decrease in planktonic growth rate (Figure 4) was reflected in a slower biofilm development of the high rhamnolipid producer although a higher biomass than the reference strain was eventually reached (Figure 5). The medium rhamnolipid producer and the reference strain follow the same biofilm development until the dispersal of the reference strain. Here-

after, the rhamnolipid producing strain continues to grow and reach 60% more biomass than the reference strain. The amount of produced rhamnolipids does have an impact on the biofilm dispersal. Although both of the rhamnolipid producing strains reached a higher biofilm biomass, the biofilm dispersal initiates earlier in the high rhamnolipid producer than the medium rhamnolipid producer. Hence, the amount of present rhamnolipids does have an impact on dispersal, however, compared to the reference strain rhamnolipid producers reach higher biofilm biomass.

Heterologous expression of rhamnolipids in *P. putida* increase swarming motility

Rhamnolipids have been shown to have an effect on both swarming motility and biofilm formation in *P. aeruginosa* (Pamp and Tolker-Nielsen, 2007), and similar motility-related effects could explain the observation of altered biofilm formation in our engineered *P. putida* strains. Indeed, we observed that heterologous expression of rhamnolipids in *P. putida* results in an increase of swarming motility and expansion of colonies on solid surfaces (Figure 6). In order to verify that the increased expansion was a result of rhamnolipids the assay was repeated in the presence of the surfactant Tween 20. This resulted in increased colony size of the reference strain. Although the colony did not become as large as the rhamnolipid producing strain it increased by more than 2 fold. The presence of Tween 20 did not have a great influence on the rhamnolipid producing strain. This could be a consequence of an abundant quantity of rhamnolipids compared to Tween 20.

Continuous production of rhamnolipids in biofilm encased *P. putida*

A continuous production of rhamnolipids using biofilm as the production platform was evaluated using flow cell technology (Tolker-Nielsen and Sternberg, 2011, Gjermansen et al., 2010). The biofilm was grown on a glass substratum and growth was followed *in situ* using confocal laser scanning microscopy. A high and a medium rhamnolipid producer were grown and the rhamnolipid content quantified and compared to the reference strain. Representative pictures of the high rhamnolipid producer (VW224) and the reference strain (VW230) are shown in Figure 7. No apparent visual difference in the biofilm morphology was observed. However, the VW224 biofilm appeared to contain more biomass. For quantitative comparison the biomass was quantified based on the obtained pictures using COMSTAT2 (Heydorn et al., 2000, Vorregaard, 2008). Figure 8A, B and C show the quantified biomass of the biofilm from flow chambers. The fluctuations observed in the quantification demonstrate the heterogeneity and complexity of biofilm. The biofilm biomass is difficult to quantify, specially for *P. putida* biofilm (Heydorn et al., 2000). Even though fluctuation occur a quantitative measure of the biomass enables comparison across the strains, particularly when combined with crystal violet assays. The biofilm stimulating effect observed in the microtiter assay (Figure 5) is also evident in flow chambers (Figure 8D). Although the biomass fluctuates the rhamnolipid production showed to be consistent and reaches a stabile level after which the production is constant for both the medium and the high rhamnolipid producer (Figure 9). The rapid achievement of maximal production titer reflects a fast and reproducible establishment of a biofilm capable of producing rhamnolipids. Both the biofilm and the rhamnolipid production were stably maintained during the cultivation period.

Discussion

The quest for replacing petrochemicals with biochemicals gives challenges in finding the optimal organism and cultivation conditions. In this study we have explored the potential of biofilms as alternative production platform for biosynthesis of chemicals which are inherently troublesome to produce in conventional bioreactor setups.

Heterologous biosynthesis and detection of rhamnolipids in *P. putida*

Although the fast and reliable method of using synthetic promoter libraries has been employed for several species (Solem and Jensen, 2002, Rud et al., 2006, Rytter et al., 2014, Sohoni et al., 2014), this is the first time this technique has been employed for *Pseudomonas* ssp. The use of SPL made it possible to investigate the impact of heterologous rhamnolipid production in *P. putida* regarding growth rate and biofilm capabilities. We selected a constitutive rather than an inducible promoter design, as the even distribution of inducers into the biofilm could be hindered by biofilm specific factors such as the extracellular matrix. Importantly, we showed that rhamnolipids promotes biofilm formation, constitutive production will not limit biofilm establishment (Figure 5 and Figure 8).

Rhamnolipid production has been investigated in both *P. putida* and *P. aeruginosa* (Ochsner et al., 1995, Wittgens et al., 2011). Many of these studies have been made by indirect quantification and by measuring the total pool of rhamnolipids, although it is well known that different rhamnolipid congeners are made resulting in different physiochemical properties (Deziel et al., 1999). We aimed to investigate the applicability of biofilm as a production platform for rhamnolipids, it was crucial to develop a method by which the different congeners could be determined in case alterations in

composition occurred as a consequence of perturbations in the intracellular metabolites. Furthermore, as production of rhamnolipids in a biofilm will result in low titers, a sensitive method for detection of small changes in rhamnolipid concentration was necessary.

With our method, we observed the fatty acid composition of the rhamnolipids to vary between C₈ to C₁₂ with C₁₀ being the most abundant (Deziel et al., 1999, Rudden et al., 2015). This is similar to the rhamnolipids produced by *P. aeruginosa* (Deziel et al., 1999, Rudden et al., 2015), and is expected due to the substrate specificity of *rhlA* from *P. aeruginosa* to C₁₀ fatty acids (Zhu and Rock, 2008). The lower limit of detection of our method was 0.09 mg/L (data not shown) which is comparable to the method recently published by (Rudden et al., 2015).

Biofilm as a production platform

Excessive foaming during rhamnolipid production in conventional bioreactors system remains a challenging problem in relation to maintaining sterility (Muller et al., 2010, Reiling et al., 1986, Urum and Pekdemir, 2004). In addition to being a problem in maintaining sterility, foam formation can also result in disturbances of the culture broth and thereby result in decreased rhamnolipid production (Reiling et al., 1986). In this study we eliminated foam production by employing biofilm encapsulated cells in a flow system as production platform.

The biofilm mode of growth represent another set of challenges not present in other bioreactor systems. For example, since rhamnolipids are biosurfactants they could have a severe impact on the biofilm formation process by dissolving the encapsulated cells. Rhamnolipids could potentially be involved in removing extracellular polymeric

substances thereby destabilising the biofilm and consequently disrupting it (Diaz De Rienzo et al., 2016). However, we found that rhamnolipids stimulated *P. putida* biofilm formation through enhanced cell motility as previously shown for *P. aeruginosa* (Pamp and Tolker-Nielsen, 2007). However, the difference in biofilm morphology was not as pronounced in our *P. putida* biofilms as observed in *P. aeruginosa* (Pamp and Tolker-Nielsen, 2007). We also observed that the amount of rhamnolipids has an effect on biofilm dispersal. The more rhamnolipids are produced the earlier the biofilm dispersal occur. The presence of rhamnolipids does however result in more biofilm biomass compared to the reference strain in all cases (Figure 5). Hence, there is an optimum for biofilm biomass and rhamnolipid content after which the biomass starts to decline.

Instability of biofilm cells has been reported in other studies as cell morphology did change during the cultivation time (Gross et al., 2010). We verified the expression of the *rhlAB* genes in the biofilm by employing the plasmid encoded *gfp* reporter for visualisation of the *in situ* biofilm. In our case no changes were observed in relation to plasmid loss (data not shown), change in morphology or decreased rhamnolipid production.

In the present study we have focused on using biofilm as a platform for producing rhamnolipids and the associated challenges. Hence, no optimisations of the cells were made for increasing the production as well as for increased biofilm formation. A candidate for increasing production could be to reduce diversion of precursors from the biosynthesis of rhamnolipids. Wittgens et al. (2011) eliminated the synthesis of polyhydroxyalkanoate as this competes for the rhamnolipid precursor HAA. This modification resulted in an increased yield. This could be the first step in order to achieve higher production titers. Wittgens et al. (2011) also showed rhamnolipid synthesis to

be growth independent and growth should be minimised for increasing the yield. We took advantage of this knowledge in using biofilm as the production platform for heterologous synthesis of rhamnolipids. The lowered growth activity of biofilm encased cells (Sternberg et al., 1999) should reflect an increased yield without hampering the biosynthesis. Another possibility is to increase productivity by increasing the precursors for rhamnolipid synthesis. This can be mediated by perturbing the intracellular energy levels and increase the ATP demand. Introducing the F₁ part of the membrane bound F₀F₁ H⁺-ATP synthase increased the glycolytic flux in *E. coli* (Koeblmann et al., 2002). This may stimulate the intracellular processes and thereby increase the synthesis of rhamnolipids by stimulating the synthesis of precursors.

Other areas for further improvements of our production platform include prevention of biofilm dispersal by genetic engineering as previously described (Gjermansen et al., 2010), and to integrate the production genes into the chromosome of the bacteria to eliminate possible loss of plasmids during production (although the employed plasmid can be stably maintained in *P. fluorescens* without selection (Heeb et al., 2000)).

Conclusion

In this study we were able to successfully utilise biofilm as a production platform. By employing SPL we characterised the effect of producing rhamnolipids in relation to growth and on biofilm capabilities of the non-pathogenic bacterium *P. putida*. The *in situ* investigation of the biofilm formation enabled invaluable exploration of the effect of producing rhamnolipids. This study adds on to the increasing knowledge of *P. putida* and the wide applicability of this organism in industrial settings. The continu-

ous production of rhamnolipids exemplified in this study show that this mode of growth can support production of troublesome biochemicals. A continuous production of biochemicals eliminate product inhibition and the inherent resistance of biofilm make it a competent candidate for producing biochemicals which are toxic as well as for using alternative feedstocks without being necessitated to make a detoxification.

Materials and methods

Strains and growth conditions

E. coli DH5 α strain was used for standard DNA manipulations. *Pseudomonas aeruginosa* PAO1 and *Pseudomonas putida* KT2440 were used for synthetic promoter library construction. The employed strains are listed in Table 1. The strains were propagated in modified Lysogeny broth (LB) medium containing 4g of NaCl/liter instead of 10 g NaCl/liter and with peptone instead of tryptone (Bertani, 1951). Biofilm experiment in flow chambers were made in modified FAB medium (Heydorn et al., 2000) supplemented with 1 mM sodium citrate. Biofilm inoculum was supplemented with 10 mM sodium citrate. Tetracycline concentration of 8 μ g/mL and 20 μ g/mL was used for *E. coli* and *P. putida*, respectively. *E. coli* and *P. aeruginosa* were incubated at 37°C and *P. putida* at 30°C.

Construction of synthetic promoter library

The reporter plasmid pVW10 was constructed by PCR amplification of *gfpmut3** using Phusion polymerase and the primers Gfp_fwd and Gfp_rev, followed by double

354 digestion of the fragment and vector pME6031 with *EcoRI* and *PstI*. These were li-
355 gated together by T4 DNA ligase.

356 Constitutive SPL of the *rhlAB* operon was constructed as described by (Solem and
357 Jensen, 2002). The SPL was based on putative rRNA promoters extracted from the
358 genome sequence of *P. putida* KT2440 (accession number: NC_002947) and *P. aeru-*
359 *ginosa* PAO1 (accession number: NC_002516). Promoters of varying strength were
360 obtained by randomisation of the nucleotides surrounding -10 and -35 consensus se-
361 quences (primers: SPL_rhlAB and rhlAB_rev). The native promoter of the *rhlAB* op-
362 eron in *P. aeruginosa* PAO1 and a promoterless *rhlAB* operon were made as reference
363 to the constructed SPL and for validation of promoter activity (primers: Na-
364 tive_rhlAB, SPL_con_rhlAB and rhlAB_rev).

365 Genomic DNA from *P. aeruginosa* PAO1 was extracted using Wizard Genomic DNA
366 Purification Kit (Promega). The *rhlAB* operon was PCR amplified from genomic *P.*
367 *aeruginosa* PAO1 using Phusion polymerase. The employed primers are listed in Ta-
368 ble 1. Following double digestion of the fragments containing *rhlAB* operon and vec-
369 tor pVW10 with *BglII* and *EcoRI* these were ligated together with T4 DNA ligase. All
370 enzymes for DNA manipulations were bought from ThermoFisher Scientific and used
371 as recommended. Primers were purchased from Integrated DNA Technologies. The
372 SPL rhlAB primer was purchased as ultramer.

373 The SPL was introduced into *P. putida* by electroporation (Choi et al., 2006) to avoid
374 biases in promoter strength by using *E. coli* as an intermediate strain. The SPL was
375 constructed in three independent rounds. *Pseudomonas putida* KT2440 containing
376 SPL were randomly picked followed by manual inspection for highly active promot-
377 ers missed in the random selection.

For biofilm formation in flow chambers the reference strain containing *P. putida* pVW10 was fluorescently tagged at an intergenic neutral chromosomal locus with *gfp* in mini-Tn7 constructs as described by (Koch et al., 2001). The rhamnolipid producing mutants were visualised by the plasmid encoded *gfp* reporter gene.

GFP analysis

The promoter strength was determined by quantification of *gfp* expression at single-cell level by flow cytometry. The *P. putida* strains containing the promoter library were grown in LB medium supplemented with tetracycline in 96-well microtiter plates. Overnight cultures of approximately 16h were diluted 50 fold and incubated for an additional 3h at 30°C with shaking at 600 rpm to ensure exponentially growth. The cultures were diluted 5 times in 0.9% NaCl before measuring *gfp* expression on a FACSCalibur (Becton Dickinson) flow cytometer. The data was analysed in FlowJo.

Rhamnolipid extraction and analysis

A mono-rhamnolipid standard was used for calibration and validation purposes (Sigma-Aldrich). Cells were removed by filtration (0.22µM) and the supernatant, 1 mL, was mixed with 2 times the volume of 70% acetonitrile. A two-phase separation was achieved by addition of a mixture of sodium sulphate and sodium chloride powder \approx 100 mg followed by centrifugation at $4500 \times g$ for 5 min (QuEChER extraction) (Anastassiades et al., 2003). Chloramphenicol (50 mg/mL in ethanol) was added to a final concentration of 10 µg/mL as internal standard. The organic phase was transferred to HPLC vials and analysed on a reverse phase UHPLC-HRMS on a maXis HD quadrupole time of flight (qTOF) mass spectrometer (Bruker Daltonics, Bremen,

Germany) connected to an Ultimate 3000 UHPLC system (Dionex, Sunnyvale, CA, USA) equipped with a 10 cm Kinetex C₁₈ column (Phenomenex Torrance, CA, USA) running a linear 10-100% gradient for 15 min at 40°C and a flow at 0.4 ml/min (Klitgaard et al., 2014). The qTOF was operated in ESI⁻ negative mode, scanning m/z 50-1300, with alternating fragmentation energy over the quadrupole of 0 and 25 eV, each for 0.25 s.

UHPLC-HRMS data analysis

Extracted ion chromatograms (± 2 mDa) of the [M-H]⁻ ions were constructed from the 0 eV volt data using Compass TargetAnalysis (version 1.3 Bruker Daltonics), which also verified the isotopic patterns (I-fit <50). The 25 eV volt data were used to verify identity of the rhamnolipids, as fragment ions of rhamnose and one fatty acid moiety could be identified by the loss of the fatty acid distant to the rhamnose molecule (Supporting Information figure S1). The retention time (± 0.02 min) was compared to an authentic standard (Sigma-Aldrich). The rhamnolipid standard contained according to the manufacture 90% mono-rhamnolipid and 5% di-rhamnolipid, each of these contained several congeners with different fatty acid chains. Each of the mono-rhamnolipid congeners were integrated, [M-H]⁻, and their fractions determined in the purchased standard. The standard contained the following fractions of congeners: 84.1% Rha-C₁₀-C₁₀, 9.7% Rha-C₈-C₁₀ to; 3.4% Rha-C₁₀-C_{12:1}, 2.5% Rha-C₁₀-C_{10:1} and 0.3% Rha-C₁₀-C₁₂,

Calibration of rhamnolipids was made in acetonitrile-water (1:1 v/v) dilutions. The peak areas of rhamnolipids were normalised to that of chloramphenicol [M-H]⁻ (10 ug/ml). The standards were made in steps of 0.2 μ g/mL from 0.5 μ g/mL to 1.7 μ g/mL

and from 1.7 µg/mL to 30 µg/mL in steps of 2 µg/mL. Limit of detection was determined as the lowest point where a peak with s/n of 15 could be detected. Limit of detection was 0.09 µg/mL. R^2 of 0.995 was obtained in the range 0.09 µg/mL to 12 µg/mL of the standard.

Swarming motility

Swarming motility was assayed in LB medium containing 20 µg/mL tetracycline and solidified with 0.5% agar. The plates were point inoculated at the surface and incubated for 24 h at 22°C.

Crystal violet biofilm assay

Quantification of biofilm formation in static microtiter dishes was made by crystal violet staining as described by (O'Toole and Kolter, 1998). Briefly, over night cultures were diluted to an OD₆₀₀ of 0.010 and inoculated in 100 µL LB supplemented with 20 µg/mL tetracycline for the specified time. The wells were emptied and washed with 0.9% NaCl followed by 15 min of staining with 0.1% crystal violet (Sigma-Aldrich). The wells were washed twice in saline and adhered crystal violet was subsequently solubilised in 96% ethanol for 15 min before quantification by spectrometry at Abs₅₉₅. Microtiter dishes were made of polystyrene (TPP Techno Plastic Products AG).

Biofilm cultivation in flow chambers

Biofilms were cultivated in three-channel flow chambers with individual channel dimensions of 1×4×40 mm covered with glass coverslip (Knittel 24x50mm) serving as substratum for biofilm formation. The system was prepared and assembled as previously described by (Tolker-Nielsen and Sternberg, 2011). Overnight cultures were made in modified FAB medium supplemented with 10 mM sodium citrate. The overnight cultures were diluted to an OD₆₀₀ of 0.010 and aliquots of 500 µL were inoculated into each channel of the flow chambers. Bacterial attachment was allowed for 1 h with the chambers turned upside down without flow. The flow systems was incubated at 22°C with a laminar flow rate of 3 mL/h obtained by a Watson Marlow 205S peristaltic pump. Modified FAB medium supplemented with 1 mM of sodium citrate was used for biofilm cultivation (Heydorn et al., 2000). Tetracycline was added to the growth medium for plasmid maintenance. Bacterial growth upstream of the flow channels were removed by cutting the affected part of the tubing under sterile conditions and reattached the tubes to the flow channel.

Microscopy and image processing

Biofilm was followed *in situ* using a Leica TCS SP5 confocal laser scanning microscope equipped with detectors and filters set for monitoring Gfp fluorescence. Images were obtained with a 63x/1.20 water objective. Images of the biofilm were taken at the specified time points. Images were all acquired from random positions at a distance of 5-10 mm from the inlet of the flow channels. Biomass of the biofilm was determined based on the acquired pictures by employing COMSTAT2 (Heydorn et al.,

2000, Vorregaard, 2008). Simulated three-dimension images were generated using the
Imaris software package (Bitplane AG).

Author contributions

V.W., L.J., A.F. and P.R.J. conceived the study and designed the research. K.F.N. and
V.W designed and conducted the UHPLC-MS analysis. V.W. performed the research
and drafted the manuscript. V.W., L.J., A.F., P.R.J. and K.F.N. analysed the data and
critically read the manuscript.

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Competing interests

The authors declare no competing interest.

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Figures

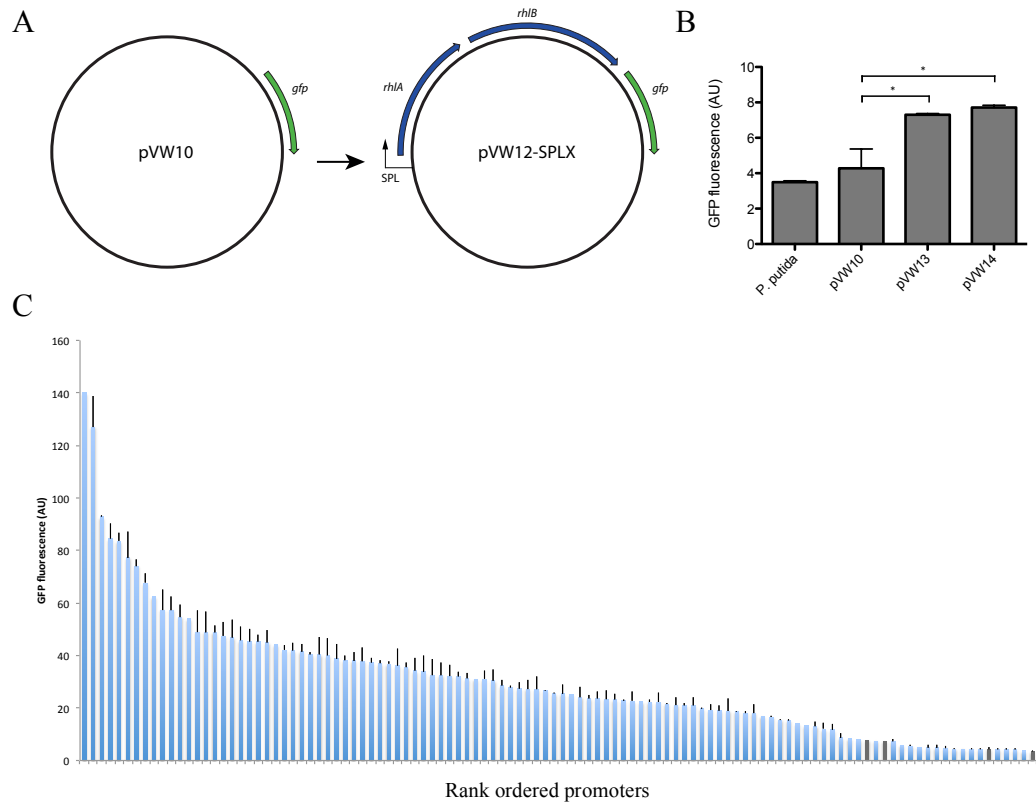


Figure 1. Construction and utilization of a synthetic promoter library in *P. putida*. (A) Outline of the plasmid construction strategy. The genes *gfp*, *rhlA* and *rhlB* indicate the green fluorescence protein, rhamnosyltransferase chain A and rhamnosyltransferase chain B, respectively. SPL indicate synthetic promoter library. The X in pVW12-SPLX represents any of the 120 synthetic promoters constructed in this study (see Materials and methods). (B) Gfp fluorescence measurement in a series of control strains without SPL. The control strains are wild type strain (*P. putida*) and strains containing an empty vector (pVW10), plasmid containing *rhlAB* operon (pVW13) with no promoter and the *rhlAB* operon with the native promoter from PAO1

661 (pVW14). The asterisks represent a significant difference ($P<0.05$). (C) The different
662 promoter strength in the constructed SPL determined as Gfp intensities. The controls
663 strains depicted in figure (C) are shown in grey. The columns represent mean value
664 and the error bars indicate standard deviation. The Gfp intensities have been replicat-
665 ed 1 to 3 times.

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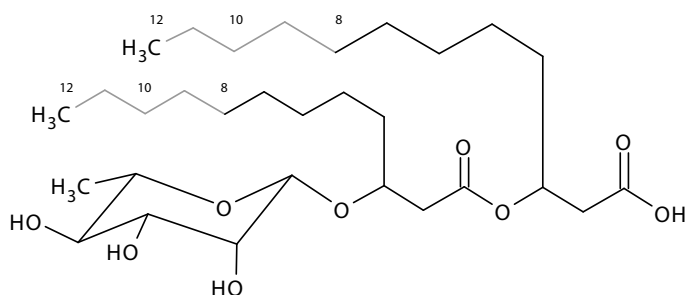


Figure 2. Outline of the different rhamnolipid congeners. The grey part of the structure is the varying part of the molecule. The numbers indicate the length of the fatty acid side chain. The most predominant congener produced by the recombinant *P. putida* is Rha-C₁₀-C₁₀. The different rhamnolipid congeners were determined based on their elemental composition.

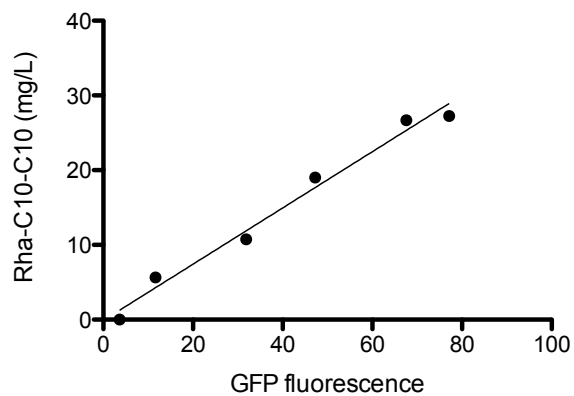
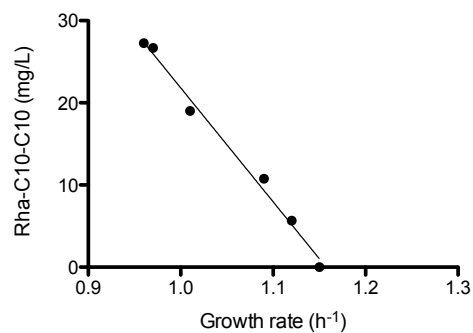


Figure 3. Correlation between *gfp* expression and Rha-C₁₀-C₁₀ concentration. The linear correlation between *gfp* and Rha-C₁₀-C₁₀ enable fluorescence to be used as an indirect measure of rhamnolipid biosynthesis.

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686 Figure 4. Correlation between rhamnolipid production and growth rate. Biosynthesis
687 of rhamnolipids has an impact on planktonic growth rate. The cells grow slower as
688 they produce more rhamnolipids.

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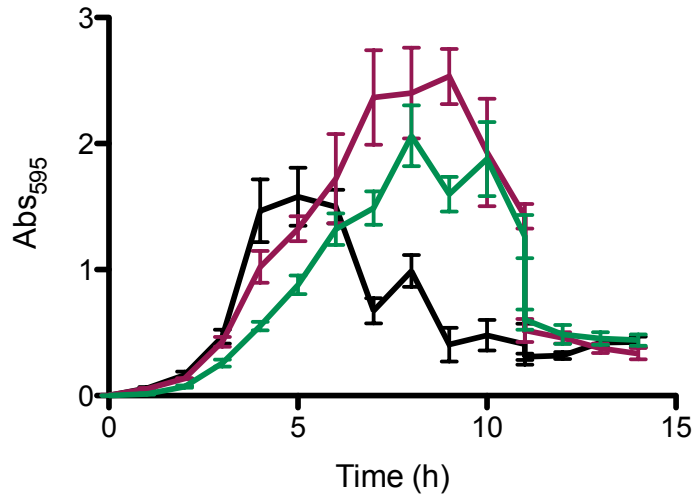


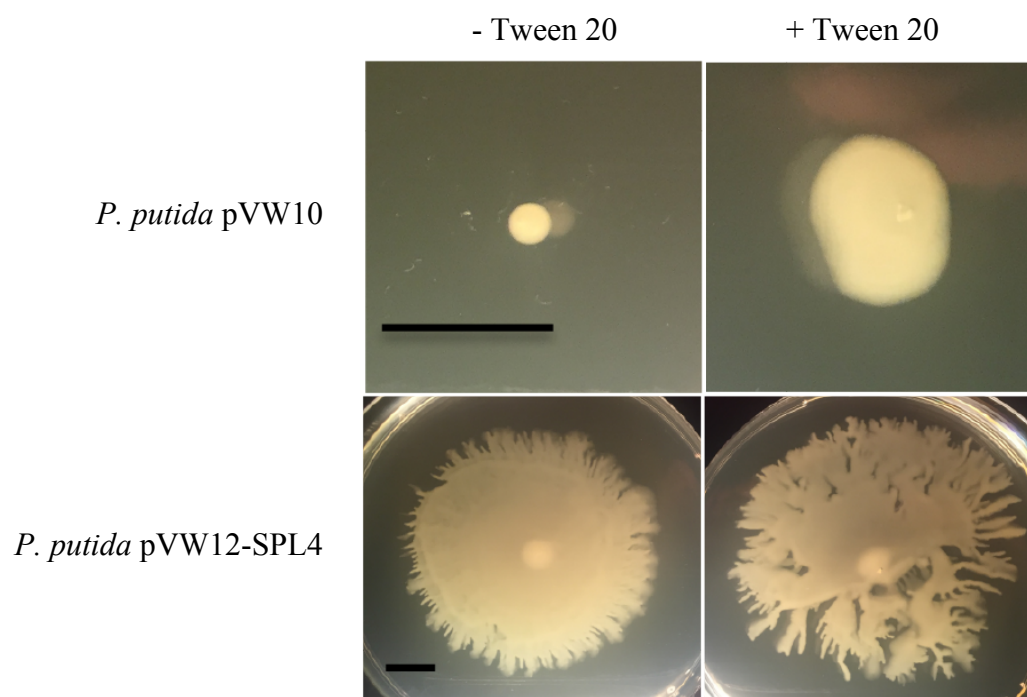
Figure 5. Biofilm development in microtiter plate assays. The amount of biofilm was quantified for the reference strain (black), a medium rhamnolipid producer (red) and a high rhamnolipid producer (green) at the specified time points. Each data point represents the mean and the error bars indicate standard deviations of eight technical replicates.

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703 Figure 6. Effect of rhamnolipid and the surfactant Tween 20 on swarming motility.

704 The top row is the reference strain and the bottom row is a rhamnolipid producer. The

705 right column has been added 0.0005% Tween 20 and the left column have not. The

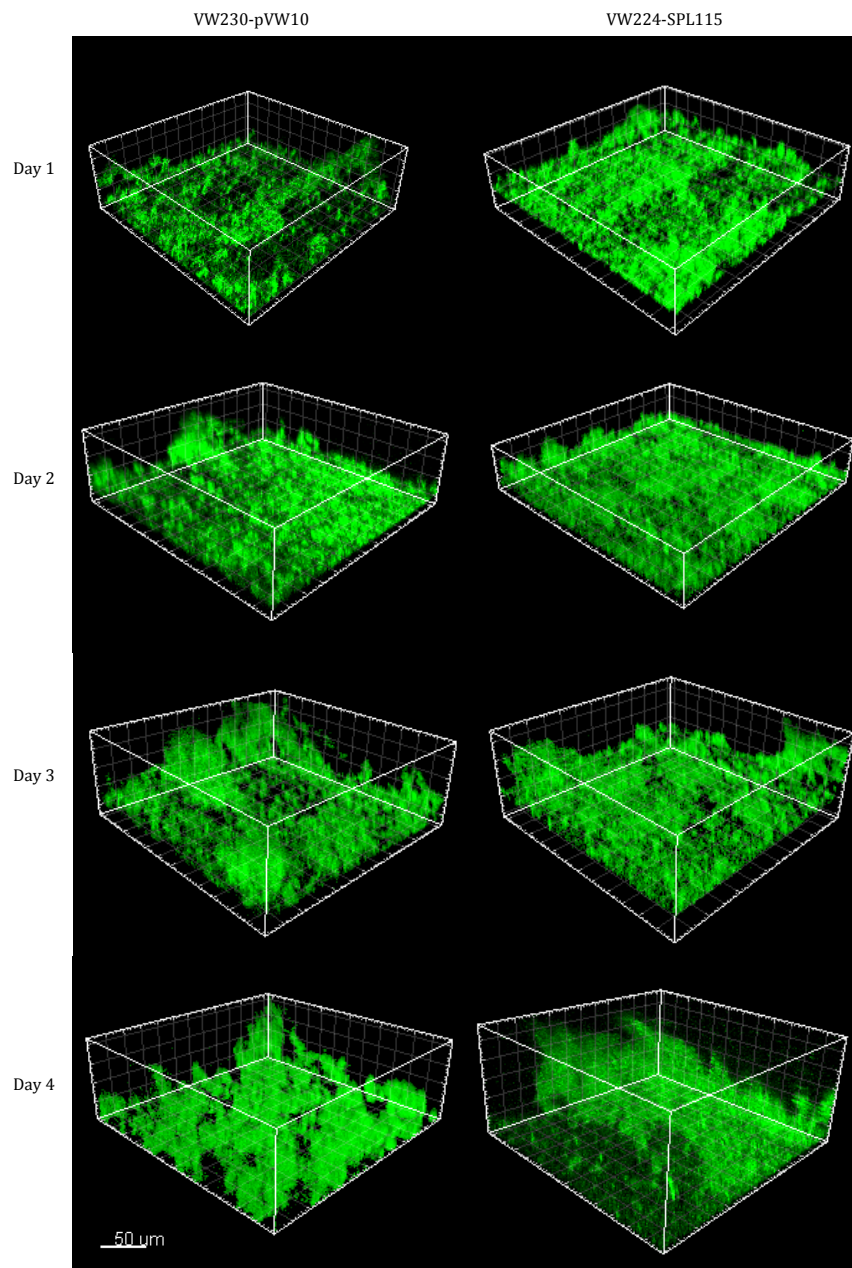
706 scale bars indicate the size of 1 cm on the pictured strain. The presence of Tween 20

707 increased the swarming motility of both strains but in particular for the reference

708 strain.

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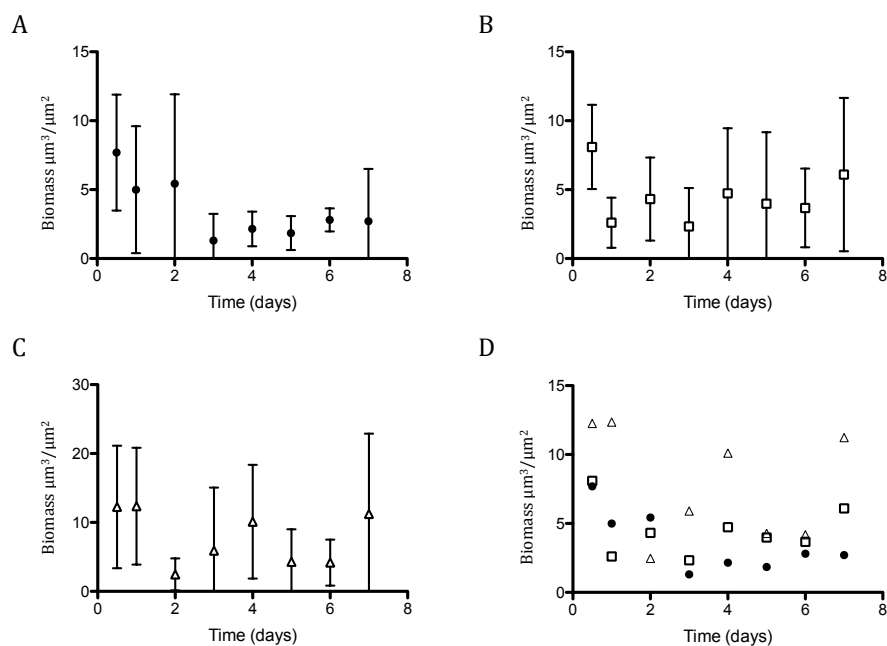
712 Figure 7. Biofilm development of a high rhamnolipid producing *P. putida* (VW224)

713 and the reference strain (VW230). Depicted are representative pictures of biofilm

714 from the specified days. The scale bar indicates the size of 50 μm .

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718 Figure 8. Biofilm biomass quantification. The biomass was quantified based on the
 719 obtained CSLM pictures. Panel A is the reference strain VW230, panel B is the medi-
 720 um rhamnolipid producer VW98, and panel C is the high rhamnolipid producer
 721 VW224. In panel D the strains are combined and only the mean values are depicted
 722 for clarity. The symbols represent the mean biomass and the error bars the standard
 723 deviation based on two biological replicates each composed of 7-11 pictures.

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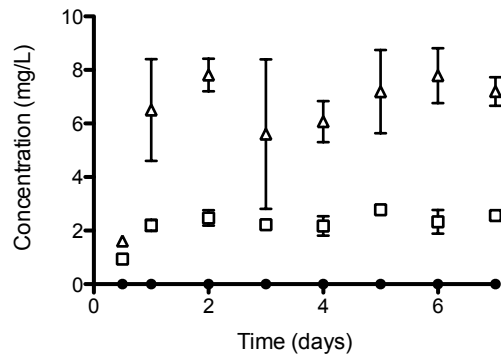


Figure 9. Continuous rhamnolipid production in a biofilm system. The reference strain (•VW230), a medium (□VW98) and a high (ΔVW224) rhamnolipid producer were cultivated for seven days and the rhamnolipid production quantified. The symbols represent mean concentration and the error bars the standard deviation of two biological replicates.

Tables

Table 1 – Strains, plasmids and primers used in this study. The restriction sites on the primers are indicated by underscore. N indicate a randomised base representing 25% of A, C, G or T.

Strain, plasmid or primer	Relevant characteristic or sequence	Reference
Strains		
<i>E. coli</i> DH5α	Host for plasmid maintenance	Laboratory strain
<i>P. putida</i>	Wild type strain; used for heterologous expression of <i>rhlAB</i> operon	(Bagdasarian et al., 1981)
<i>P. aeruginosa</i>	Wild type strain; used for amplification of <i>rhlAB</i> operon	(Holloway, 1955)
Plasmids		
pBK-miniTn7- <i>gfp2</i>	Gm ^r ; P _{rmB} P1 <i>gfp</i> AGA Delivery vector for miniTn7- <i>gfp2</i>	(Koch et al., 2001)
pRK600	Cm ^r ; <i>ori</i> ColE1 RK2- <i>mob</i> ⁺ RK2- <i>tra</i> ⁺	(Kessler et al., 1992)
pUX-BF13	Ap ^r ; <i>mob</i> ⁺ <i>ori</i> R6K	(Bao et al., 1991)
pJBA27	Helper plasmid in mating Helper plasmid providing Tn7 transposition function in trans <i>gfp</i> mut3*	(Andersen et al., 1998)
pME6031	Tc ^r ; derivate of pME6010; clonings vector maintained in <i>Pseudomonas</i> strains without selection pressure	(Heeb et al., 2000)
pVW10	pME6031:: <i>gfp</i> mut3*	This study
pVW13	pVW10 with promoterless <i>rhlAB</i>	This study
pVW14	pVW10 with <i>rhlAB</i> and its native promoter	This study
pVW12-SPL1 to pVW12-SPL120	SPL in front of <i>rhlAB</i>	This study
Primers		
Gfp Fwd	5'-AGATAGAATTCAGATTCAATTGTGAGCGG-3'	
Gfp rev	5'-AGATACTGCAGTATCAACAGGAGTCCAAGC-3'	
SPL <i>rhlAB</i>	5'-AGATAAGATCTACN ₁₀ TTGACAN ₁₆ TATAATN ₆ CCCG-ATCGGCTACGCGTGAACA-3'	
<i>rhlAB</i> rev	5'-AGATAGAATTCATCACAGCAGAATTGGCCC-3'	
SPL con <i>rhlAB</i>	5'-AGATAAGATCTATCGGCTACGCGTGAACA-3'	
Native <i>rhlAB</i>	5'-AGATAAGATCTACCCTGCCAAAAGCCTGA-3'	

1 **Supporting information for**

2 **Biofilm as a production platform for heterologous production**

3 **of rhamnolipids by the non-pathogenic strain *Pseudomonas***

4 ***putida* KT2440**

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Rhamnolipid identification

Identification of the rhamnolipid congeners was based on their elementary composition. For identification of any possible rhamnolipid congeners in a sample, a list containing different combinations of rhamnose and fatty acids with varying chain length and saturation was used for screening the chromatograms (Table S 1). However, the engineered *P. putida* host only produced a few different congeners (Figure S 1A). The chromatograms are based on the obtained mass spectrum (Figure S 1B) which shows the determined m/z values.

The abovementioned screening method suffers from the lack of ability to discriminate between isomers, e.g. between Rha-C₁₂-C₁₀ and Rha-C₁₀-C₁₂. For this reason pseudo MS/HRMS (MS-E) was made for structural identification of the different congeners. The fragmentation pattern of the rhamnolipid congeners revealed the fatty acid composition and was used for the identification (Table S 1 and Figure S 1C,D). An example of fragmentation is shown for Rha-C₁₀-C₁₀ in Figure S 1D. The fragmentation point is indicated resulting in a m/z 333.1913 ion which is apparent in the 25 eV mass spectrum together with the lost fatty acid moiety of m/z 169.1239. In this way both the length of the fatty acid and their mutual position was elucidated. The obtained results correspond with previous results (Rudden et al., 2015, Deziel et al., 2000).

36 **Reference List**

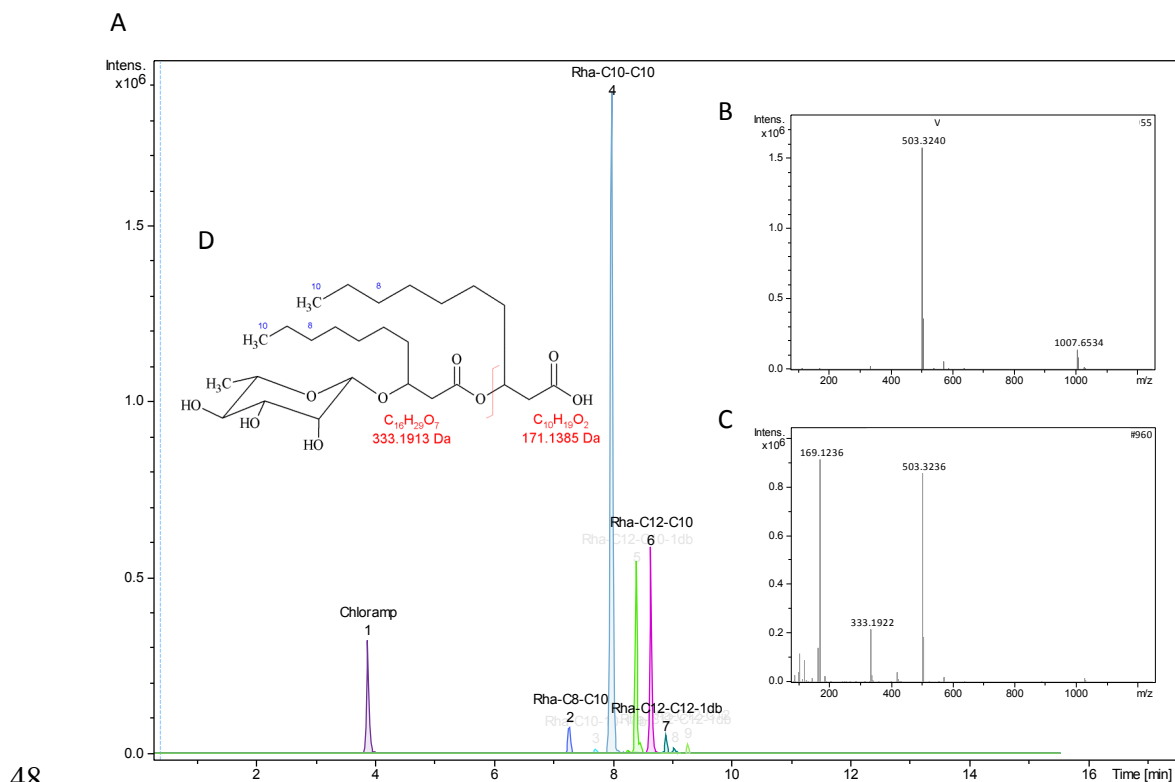
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45

46

47 Figure



49 Figure S 1 – UHPLC-HRMS analysis of rhamnolipids. Picture A is the extracted ion
 50 chromatogram of the internal standards and the four most abundant rhamnolipid con-
 51 geners from the engineered *P. putida* strain VW224. The first peak is chlorampheni-
 52 col followed by the rhamnolipid congeners Rha-C₈-C₁₀, Rha-C₁₀-C₁₀, Rha-C₁₀-C₁₂:1
 53 and Rha-C₁₂-C₁₂. The full scan mass spectrum of the most predominant rhamnolipid
 54 congener Rha-C₁₀-C₁₀ is shown in figure B. The 25 eV mass spectrum of Rha-C₁₀-C₁₀
 55 is depicted in figure C. In figure C the [M-H]⁻ ion and the fragmented ion can be seen
 56 from the pseudo MS/MS analysis. An example of the fragmentation pattern is shown
 57 in figure D for Rha-C₁₀-C₁₀. The breaking point and the resulting fragment masses are
 58 indicated in red.

59

60

61 **Table**

62 Table S 1 – The rhamnolipid congeners screened in the chromatograms. Pseudomo-
 63 lecular ions and fragmentation ions used for structural identification of the different
 64 rhamnolipid congeners is shown. The MS/MS ions are the fragmented ions. The first
 65 ion is the rhamnose and one fatty acid residue and the next is the fatty acid ion. The
 66 ions marked with * could be identified in the MS/MS (25eV mass spectrum). The re-
 67 maining ions could not be identified due to the absence of the congener or very low
 68 presence.

Compound	MS [M-H]⁻	MS/MS [M-H]⁻
Rha-C ₁₀ -C ₁₀	503.3226	333.1925* - 169.1239*
Rha-C ₁₀ -C ₁₂	531.3539	333.1925* - 197.1549*
Rha-C ₁₀ -C _{12:1}	529.3382	333.1925* - 195.1390*
Rha-C ₈ -C ₁₀	475.2913	305.1603* - 169.1239*
Rha-C ₁₂ -C _{12:1}	557.3695	360.2148 – 197.1542
Rha-C ₁₂ -C ₁₂	559.3852	360.2148 – 199.1698
Rha-C ₁₀ -C _{10:1}	501.3069	333.1925 – 167.1083
Rha-C ₈ -C ₈	447.2594	305.1603 – 143.1072
Rha-C ₈ -C _{8:1}	445.2437	305.1603 – 141.0916
Rha-C ₈ -C _{10:1}	473.2750	305.1603 – 169.1229

69

Paper 2

Glycerol adapted *Pseudomonas putida* with increased ability to proliferate on glycerol

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Manuscript in preparation.

1 **Glycerol adapted *Pseudomonas putida* with increased ability**
2 **to proliferate on glycerol**

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15 Keywords: *Pseudomonas putida* KT2440, biofilm, glycerol metabolism, adaptive
16 evolution

Abstract

A transition toward renewable resources is needed in order to obtain sustainable production of chemicals. One such alternative feedstock is the waste material crude glycerol generated from the biodiesel production. The utilisation of this abundant low cost feedstock is desirable in reducing the production costs. The exploitation is however often associated with hampered microbial growth owing to the residual impurities from the transesterification process generating biodiesel. The bacterium *Pseudomonas putida* has shown not to be severely affected by these impurities. Glycerol does however not provide substantial proliferation of *Pseudomonas putida*. We improved glycerol utilisation in this study by making adaptive evolutionary engineering. The evolved strains exhibited improvements in all investigated parameters with up to 148% increased growth rate, 57% increased final cell density and 11% reduction of lag phase. In five out of six evolved lineages we observed genomic alterations in the *roxS* gene indicating its importance in improved glycerol capabilities. Furthermore, we demonstrate that glycerol can support surface-associated biofilm formation. This study point towards using glycerol as a low cost feedstock for both planktonic and biofilm based production platforms.

Introduction

A lot of effort is being made in order to reduce the extensive use of fossil fuels. Different alternatives to these resources are being considered for the purpose of replacing them with sustainable feedstocks. In recent years biodiesel has become an established alternative to conventional fuels as it can be used in conventional diesel engines with little or no major modifications (Johnson and Taconi, 2007).

42 Production of biodiesel generates crude glycerol as a waste product accounting
43 around 10 wt% of the produced biodiesel (Johnson and Taconi, 2007). This has re-
44 sulted in a major increase of crude glycerol as the biodiesel production has continued
45 to increase. The concomitant accumulation of crude glycerol has made it an attractive
46 low cost substrate for microbial cell factories (da Silva et al., 2009). The use of crude
47 glycerol as a feedstock is however associated with challenges as it contains various
48 impurities such as methanol, salt ions and heavy metals from the transesterification
49 reaction which hampers microbial growth (da Silva et al., 2009, Hu et al., 2012, Fu et
50 al., 2015). Nonetheless, the use of crude glycerol as an alternative feedstock will im-
51 prove the cost compatibility of biodiesel as well as the biochemicals produced by the
52 microbial cell factories (Johnson and Taconi, 2007).

53 The applicability of crude glycerol as a feedstock has been investigated in various
54 studies (Fu et al., 2015, Verhoef et al., 2014, Fu et al., 2014). Some organisms are
55 able to cope with the challenges associated to the present impurities in crude glycerol
56 while other organisms are severely hampered. For example, Verhoef et al. (2014)
57 showed that *Pseudomonas putida* is particularly tolerant towards the impurities in
58 crude glycerol. In addition to this *P. putida* also possess high tolerance toward various
59 solvents which is beneficial in connection to microbial production of biochemicals
60 (Poblete-Castro et al., 2012). Furthermore, *P. putida* can grow in surface-associated
61 biofilm which offers an alternative to the conventional bioreactor systems (Rosche et
62 al., 2009). This mode of growth is known to be more tolerant towards toxic com-
63 pounds than the planktonic mode of growth (Li et al., 2006).

64 These characteristics suggest that *P. putida* is a potentially attractive candidate in
65 connection to biofilm-based cell factories that use crude glycerol as feedstock. The
66 challenge of using glycerol is the limited knowledge on glycerol metabolism in *P.*

putida. Furthermore, the use of glycerol is associated with both low growth rate and long lag phase (Nikel et al., 2014). This is a major limitation in exploiting this compound. Hence, in this study we sought to improve *P. putida* growth on glycerol. This was achieved by employing adaptive laboratory evolution (Meijnen et al., 2008). In this study six lineages of *P. putida* was evolved which all had a significantly increased growth rate. The evolved strains were genome sequenced and the mutations identified. We evaluated the use of glycerol as an alternative substrate for both planktonic and biofilm cultivation. Since the composition of crude glycerol can vary depending on origin of production (Thompson and He, 2006, Hu et al., 2012) reagent grade glycerol was used in this study in order to ascribe the observations to the glycerol metabolism without being influenced by the contaminants.

Results

***Pseudomonas putida* forms biofilms when cultivated on glycerol**

Biofilm as a production platform have attracted increasing attention in recent years (Qureshi et al., 2005, Rosche et al., 2009). So far, *Pseudomonas putida* biofilm formation has primarily been investigated in rich media or using citrate as carbon source (Gjermansen et al., 2010, Yousef-Coronado et al., 2011, Lopez-Sanchez et al., 2013). To expand the usability of glycerol as a low cost feedstock in connection to biofilm based cell factories, we investigated the ability of glycerol to sustain biofilm formation. To this end, we monitored biofilm development *in situ* of *P. putida* KT2440 using both citrate and glycerol as carbon source (Figure 1). By comparing glycerol grown biofilm and citrate grown biofilm it is evident that glycerol can support biofilm

growth. Initially, less biofilm biomass was present on glycerol compared to citrate, but on the second day comparable amount of biomass was reached.

Evolution of glycerol-adapted *Pseudomonas putida* variants

To improve glycerol utilisation of *P. putida* an adaptive evolution experiment was performed. Initial experiments were made in order to determine a suitable minimal medium. Based on these results MOPS minimal medium was favoured compared to AB10 and MSN minimal media (see Materials and methods), since this medium supported high growth rate, more homogeneous cultures and higher cell densities (Figure 2). Different concentrations of glycerol were also explored. We chose the highest concentration of glycerol at which growth rate was not severely disturbed (Figure 2A) in order to achieve high cell densities at the end of growth (Figure 2B). Based on these preliminary studies the MOPS minimal medium supplemented with 3% glycerol was chosen for the adaptive evolution experiment.

Six parallel lineages were prepared from single colonies and propagated by serial transfers into fresh medium generating approximately 700 generations of evolved strains. This experimental approach was chosen, as limited knowledge is present on glycerol metabolism in *P. putida*. From each of the evolved lineages a single colony was chosen for characterisation and genome sequencing. It was evident that all six lineages had adapted to glycerol utilisation by improving various parameters. As listed in Table 2 all evolved strains had increased growth rate, higher final cell density and a shorter lag phase. Importantly, all of these parameters were significantly improved in the evolved strains demonstrating the feasibility of this approach to improve glycerol utilisation of *P. putida*. The enhanced growth rate observed in the evolved strains is approaching the growth rate in rich medium such as LB (in LB μ is 1.7 h^{-1}).

We next sought to elucidate the genomic alterations underlying these improvements in growth. Whole-genome sequencing of six variants (one from each of the six evolved lineages) enabled the identification of nucleotide substitutions and insertions/deletions that accumulated during the experiment (Table 3). Overall, we found between three and five genomic alterations per variant strain (Table 3). Importantly, the genomic analysis revealed several examples of the same genes acquiring mutations in multiple lineages. Such example of parallel evolution is a strong indicator that the mutations are beneficial in the particular selective environment. Especially, the *roxS* gene is interesting as five out of six lineages have alterations in this gene. Three lineages had a six base pair deletion, one with two base pair substitution and one with a single base pair substitution. Hence, this gene seems to be very important in mediating the observed improvements (Table 2).

Discussion

Glycerol metabolism is not well known in *P. putida*. Since this compound is an abundant low cost substrate we wanted to investigate the applicability of glycerol as a feedstock. Crude glycerol has been used in various studies for producing biochemicals (Fu et al., 2014, Verhoef et al., 2014). However, growth on glycerol is slow combined with long lag phase making its use cumbersome (Nikel et al., 2014). In this study we have improved the growth on glycerol and explored the usability of glycerol for both planktonic growth as well as biofilm growth.

The increasing knowledge of biofilm formation has made it an attractive candidate for industrial applications. Recently several studies have been published on using biofilm as the production platform for biochemicals (Gross et al., 2010, Li et al., 2006). The

139 studies on biofilm formation primarily use rich media, glucose or citrate as the carbon
140 source. For achieving cost competitive biochemicals produced from biofilm we have
141 explored the use of glycerol as the substrate. We were able to demonstrate that glycerol
142 can support *P. putida* biofilm growth. The biomass of the established biofilm is
143 comparable to that of citrate (Figure 1).

145 **Glycerol evolved *Pseudomonas putida* strains**

146 In order to improve growth on glycerol we made an adaptive evolution experiment in
147 this study. The evolved strains clearly had a growth advantage compared to the reference
148 strain (Table 2). In addition to their increased growth rate the evolved lineages
149 all had a higher final cell density. This indicates better utilisation of the substrate.

150 The identified mutations resulting in the altered changes point toward parallel evolution.
151 Many of the same genes were mutated in several lineages. The *roxS* seems to be
152 very important in adapting to glycerol. It encodes a sensor histidine kinase which is
153 part of a two component system together with a response regulator (PP_0888) placed
154 downstream of the locus. Since it is part of a regulatory system changes in the functionality
155 will result in pleiotropic effects. The RoxS/RoxR regulon have been shown
156 to be involved in various process such as metabolism and respiratory responses
157 (Fernandez-Pinar et al., 2008). In particular it has been shown as a key sensor/
158 regulator of the redox state in *P. putida* (Fernandez-Pinar et al., 2008).

159 The SMART analysis was employed to locate the mutations in order to determined if
160 they are present in any domains. The deletion, which occurs in 3 lineages, is placed
161 upstream of the histidine kinase domain which also is the case of the two base pair
162 substitution lineage (VWgly5). In the last lineage (VWgly7) the mutation results in an

amino acid substitution inside of the histidine kinase domain. This may result in altered functionality and result in conformational changes.

Mutation in the *lapA* gene appears in two of the lineages. This gene encodes a surface adhesion protein which is involved in biofilm formation. Interestingly, this mutation appears in the only lineage (VWgly4) which does not contain any alterations in the *roxS* gene. In fact lineage VWgly7 having both *roxS* and *lapA* mutations is the lineage with the lowest growth rate whereas VWgly4 has a significantly higher growth rate ($P<0.05$) and is among the highest achieved.

Glycerol as a substrate for biofilm development

The glycerol evolved strains exhibit significant improvements in planktonic growth compared to the reference strain. However, the mutations given rise to the improvements may not be beneficial for supporting biofilm growth. Fernandez-Pinar et al. (2008) show that biofilm capabilities of a *roxS* mutant is highly dependent on the substratum. They observed a significantly lowered biofilm formation on corn seeds by a *roxS* mutant but this difference was abolished on an abiotic surface. Hence, the choice of substratum will be critical for establishing biofilm of the glycerol evolved strains. Interestingly, one of the lineages (VWgly7) harbours a mutation in the *lapA* gene together with *roxS*. The *lapA* gene encodes a large adhesive protein involved in biofilm formation. Gjermansen et al. (2010) showed that a *lapA* mutant is unable to produce biofilm. Since either a *roxS* or *lapA* mutation is apparent in all of the glycerol evolved lineages, the use of these strains as a biofilm platform could be impossible if not the surface is carefully selected.

The presence of these mutations indicates that the enhanced growth abilities on glycerol may come with the cost of impaired biofilm formation capabilities. One possibil-

ity of circumventing this hurdle could be by using an appropriate abiotic substratum. However, this needs to be elucidated. Interestingly, none of the observed mutation appeared in the *glp* regulon which is important in glycerol metabolism (Nikel et al., 2014). Since mutations in regulatory genes, such as *roxS*, results in pleiotropic changes the molecular mechanism remains to be elucidated. Nonetheless, the changes somehow result in enhanced glycerol metabolism.

Conclusions

The results presented in this study show that glycerol can successfully be used as a substrate for both planktonic and biofilm growth. By using adaptive evolution we were able to evolve strains capable of utilising glycerol to a much better extent than the reference strain. The enhanced performance on glycerol does however seem to come on the expense of biofilm impairments. Further characterisations of the glycerol evolved strains is needed for definite conclusions.

Materials and methods

Strains and growth condition

The growth performance of *Pseudomonas putida* KT2440 was evaluated in the following minimal media: AB10, minimal salt medium (MSM) and morpholinepropane-sulfonic acid (MOSP) buffered medium (Nielsen et al., 2000, Deziel et al., 2003, Jensen and Hammer, 1993). These media were supplemented with different concentrations of reagent grade glycerol (Sigma-Aldrich). The optimal medium was determined as MOSP medium supplemented with 3% glycerol. This was used for the adaptive laboratory evolution experiment.

The evolution experiment was made in six parallel lineages each started from a single colony. The lineages were serially transferred each day from an over night culture diluted 100 fold into fresh MOPS medium supplemented with 3% glycerol for 15 weeks. This resulted in approximately 700 generations of evolution. A single colony from each of the evolved lineages was isolated for growth characterisation and whole-genome sequencing.

Growth experiments were made in shake flasks and in microtiter dishes (TPP Techno Plastic Products AG). The optimal minimal media and glycerol concentration was determined in shake flask experiments by diluting the over night cultures to a start OD₆₀₀ of 0.01. The glycerol evolved strains were grown in MOPS medium supplemented with 3% glycerol. Over night cultures were diluted to OD₆₀₀ of 0.001 and distributed into microtiter dishes and sealed with a permeable membrane (Breathe-Easy, Sigma-Aldrich). The microtiter dish growth experiment was made in Cytation 5 imaging reader (BioTek, Holm & Halby, Denmark). The temperature was set to 30°C with continuous shaking (3 mm) and OD₆₀₀ measurements every 5 min.

For visualising biofilm growth *in situ* the strains were chromosomally tagged at an intergenic neutral locus with *gfp* in a mini-Tn7 construct as described by Koch et al. (2001). The employed strains are listed in Table 1.

Genome sequencing

The glycerol evolved strains were genome sequenced to identify the alterations which have resulted in the increased growth rate. Single colonies from the evolved strains were isolated and used for genome sequencing. Genomic DNA was extracted using Wizard Genomic DNA Purification Kit (Promega). Library preparation and DNA sequencing (paired end) was made by Beijing Genomics Institute (BGI) in Hong Kong.

237

238 **Data analysis**

239 Data analysis of the sequenced genomes was made in CLC genomic workbench (Aar-
240 hus, Denmark). The paired end reads were mapped to the reference genome (acces-
241 sion number: NC_02947) using the default settings. Variant calling was made to the
242 reference genome followed by comparison of the evolved glycerol strains to the refer-
243 ence strain used for the adaptive laboratory evolution experiments. The default pa-
244 rameters were used for variant calling. The determined single nucleotide polymor-
245 phisms and insertion/deletion were filtered to remove alterations which did not appear
246 in both forward and reverse reads.

247

248 **Biofilm cultivation in flow chambers**

249 Biofilm cultivation was made in three-channel flow chambers with individual channel
250 dimensions of 1×4×40 mm covered with a glass cover slide (Knittel 24x50mm). The
251 glass cover slide served as substratum for biofilm formation. The system was pre-
252 pared and assembled as described by Tolker-Nielsen and Sternberg (2011). Overnight
253 cultures were made in modified FAB medium (Heydorn et al., 2000) supplemented
254 with 5 mM glycerol or sodium citrate. Over night cultures were diluted to OD₆₀₀ of
255 0.010 and 500 µL of the diluted culture was injected into the channels. The flow
256 chambers were turned upside down for 1 h to allow bacterial attachment without flow.
257 The biofilm system was incubated at 22°C with modified FAB medium supplemented
258 with 0.5 mM glycerol or sodium citrate. A laminar flow rate of 3 mL/h obtained by a
259 Watson Marlow 205S peristaltic pump.

260

Microscopy and image processing

The biofilm formation was followed *in situ* using a Leica TCS SP5 confocal laser scanning microscope equipped with detectors and filters set for monitoring Gfp fluorescence. Biofilm was magnified by a 63x/1.20 water objective. Biofilm images were acquired at the specified time points at a random position at a distance of 5-10 mm from the inlet of the flow channels. Three-dimension images were generated using the Imaris software package (Bitplane AG).

Figures

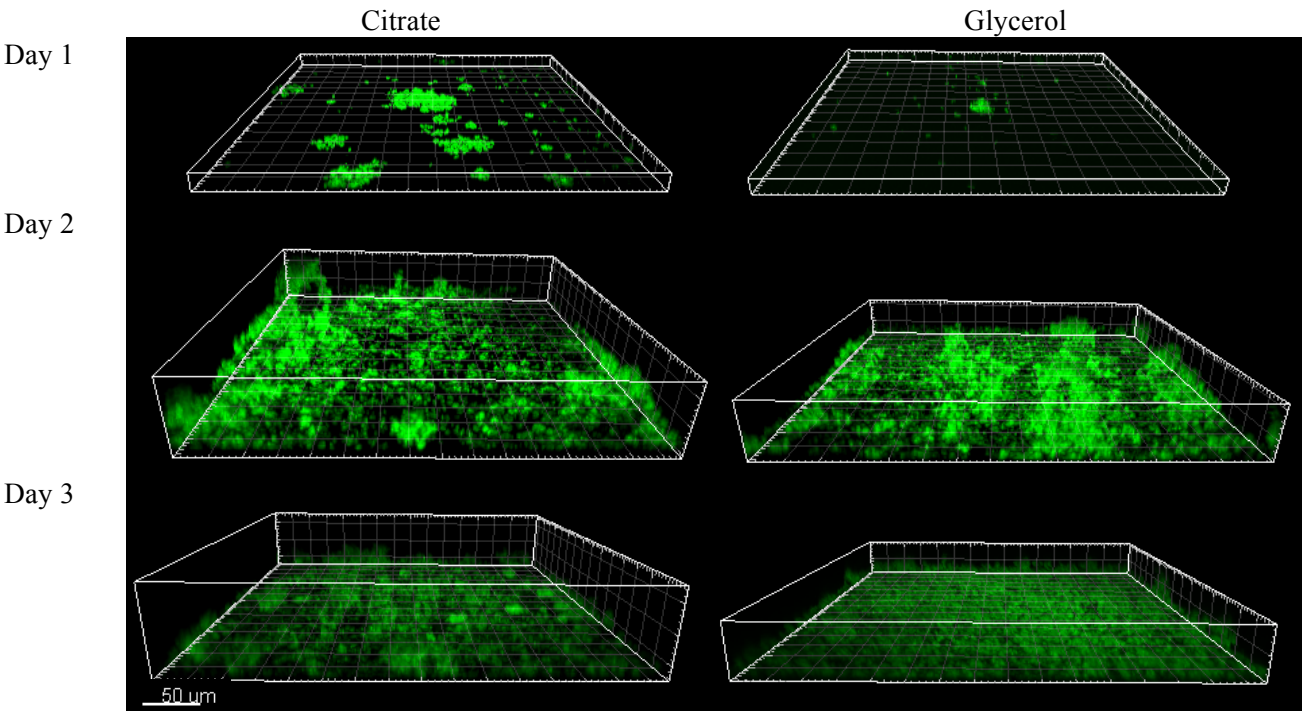


Figure 1. Biofilm formation of the reference strain *P. putida* grown on citrate or glycerol as the carbon source. Depicted are representative pictures of biofilms from the specified days. The scale bar indicates the size of 50 μm.

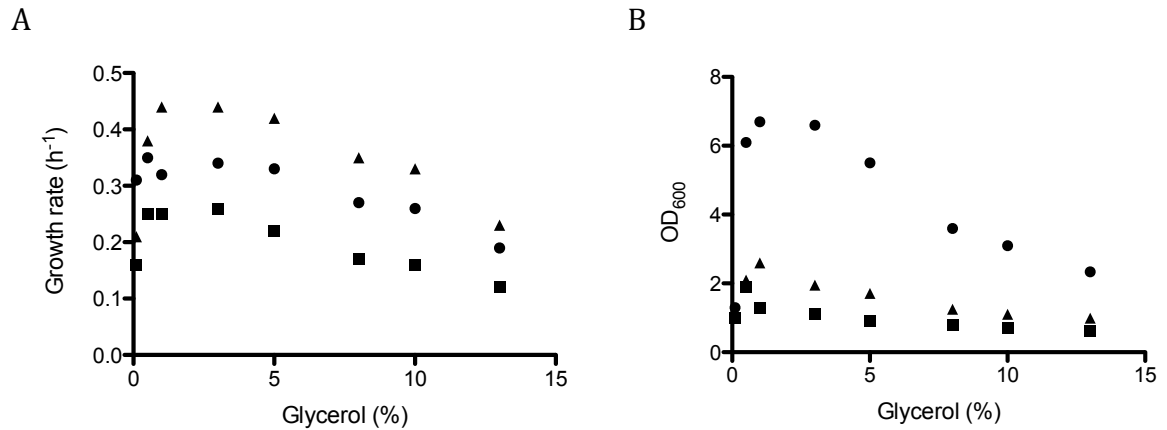


Figure 2. Comparison of growth rate and cell density in different media. The growth rate (A) and highest obtained OD₆₀₀ (B) of *P. putida* was compared in three different minimal media MOPS (circle), MSM (square) and AB10 (triangle) with varying concentration of glycerol. The growth experiment was made in shake flasks.

Tables

Table 1. Strains and plasmids used in this study.

Strain and plasmids	Relevant characteristics	Reference
Strains		
<i>P. putida</i>		Bagdasarian et al. (1981)
Plasmids		
pBK-miniTn7- <i>gfp2</i>	Gm ^r ; P _{mb} P1 <i>gfp</i> AGA Delivery vector for miniTn7- <i>gfp2</i>	Koch et al. (2001)
pRK600	Cm ^r ; <i>ori</i> ColE1 RK2- <i>mob</i> ⁺ RK2- <i>tra</i> ⁺ Helper plasmid in mating	Kessler et al. (1992)
pUX-BF13	Ap ^r ; <i>mob</i> ⁺ <i>ori</i> R6K Helper plasmid providing Tn7 transposition function in trans	Bao et al. (1991)

Table 2. Growth parameters from microtiter dish cultivation. The stated values are average and standard deviation of six technical replicates. All parameter are significantly ($P < 0.05$) different from the reference strain *P. putida* KT2440.

Strain	μ (h ⁻¹)	Max OD ₆₀₀	Lag phase ^a (h)
<i>P. putida</i> KT2440	0.48 ± 0.06	0.30 ± 0.01	16.46 ± 0.34
VWgly2	1.13 ± 0.11	0.47 ± 0.02	15.16 ± 0.11
VWgly3	1.00 ± 0.10	0.47 ± 0.02	16.01 ± 0.32
VWgly4	1.15 ± 0.02	0.45 ± 0.01	14.65 ± 0.23
VWgly5	1.19 ± 0.07	0.47 ± 0.01	14.57 ± 0.07
VWgly6	1.12 ± 0.17	0.45 ± 0.02	15.48 ± 0.52
VWgly7	0.96 ± 0.12	0.44 ± 0.01	15.04 ± 0.86

289 ^a The lag phase was determined as ceased if OD₆₀₀ reached above 0.01. This value was chosen as low
290 OD₆₀₀ are subjected to fluctuations.

291 Table 3. List of mutations in the glycerol evolved strains compared to the reference strain.

	Region	Type	Reference ^a	Allele ^b	Gene number ^c	Gene name	AA change	Non-synonymous (Yes/No)
VWgly2	1030228..1030233	Deletion	CCTGCG	-	PP_0887	<i>roxS</i>	Ala96_Arg98	Y
	1474892	SNV	T	C	IR			
	4618828	SNV	C	T	IR			
	5537487	SNV	G	A	PP_4870		Ser29Leu	Y
	6029588	SNV	C	G	IR			
VWgly3	1030228..1030233	Deletion	CCTGCG	-	PP_0887	<i>roxS</i>	Ala96_Arg98	y
	1067413	SNV	C	T	PP_0925		Ala219Val	Y
	3698879	SNV	A	T	PP_3264		Leu264Gln	Y
	5394274	SNV	G	A	PP_4740	<i>hsdR</i>		N
VWgly4	219529	SNV	G	A	PP_0168	<i>lapA</i>	Glu834Gly	Y
	4809961	SNV	G	A	PP_4235	<i>dsbD-2</i>	Gly506Asp	Y
	5681407^5681408	Insertion	-	CGGGG	IR			
	6029588	SNV	C	G	IR			
VWgly5	699720	SNV	A	G	IR			
	1030180..1030181	MNV	AC	CT	PP_0887	<i>roxS</i>		N
	2159376	SNV	A	C	PP_1916	<i>fabF</i>	Thr121Pro	Y
	5681407^5681408	Insertion	-	CGGGG	IR			
VWgly6	1030228..1030233	Deletion	CCTGCG	-	PP_0887	<i>roxS</i>	Ala96_Arg98	Y
	5681407^5681408	Insertion	-	CGGGG	IR			
	6029588	SNV	C	G	IR			
VWgly7	219529	SNV	G	A	PP_0168	<i>lapA</i>	Glu834Gly	Y

	6029614	SNV	G	C	IR
292	^a The nucleotide(s) in the reference strain which have changed. ^b The changed nucleotide(s) in the evolved strains. ^c IR, intergenic region.				

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Paper 3

Utilization and control of ecological interactions in polymicrobial infections and community-based microbial cell factories

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Utilization and control of ecological interactions in polymicrobial infections and community-based microbial cell factories.

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Abstract

Microbial activities are most often shaped by interactions between co-existing microbes within mixed-species communities. Dissection of the molecular mechanisms of species interactions within communities is a central question in microbial ecology, and our ability to engineer and control microbial communities depend to a large extent on our knowledge of these interactions. This review highlights the recent advances in relation to molecular characterization of microbe-microbe interactions that modulate community structure, activity and stability, and aims to illustrate how these findings have helped reaching an engineering-level understanding of microbial communities in relation to both human health and industrial biotechnology.

Main text

Most microbial species are embedded within ecological communities containing many species that interact with one another and their physical environment. Virtually all important microbial activities are shaped by interactions between co-existing microbes within mixed-species communities. These interactions (e.g. in the form of physical, chemical and genetic signals such as cell-cell contact [1], metabolite exchange [2] and horizontal gene transfer [3] control synergistic, antagonistic or neutral relationships among the interacting partners, and are thus responsible for overall community properties such as species composition and function. In addition, microbial interactions may be dynamic and dependent on environmental context, and microbial communities can have different spatial interactive distributions ranging from metabolic interactions between unassociated planktonic cells in the ocean [4] and long-distance electrical signalling within microbial communities [5, 6] to local cell-cell interactions occurring within surface-attached biofilms [7]. Furthermore, a series of recent studies have shown that microbe-microbe and microbe-host interactions can also be mediated by small, air transmittable molecules [8-10]. Given this complexity among microbial interactive processes, it remains a central challenge to improve our understanding of both the molecular mechanisms underlying these interaction processes, their combinatorial effects, and how these interactions ultimately modulate diversity, behaviours and activities of the individual species within complex microbial communities.

Dissection of the molecular mechanisms of species interactions within communities is an important question in microbial ecology. Recently, studies of a diverse range of microbial ecosystems have provided new insight into this area by combining omics methods with classical microbiology cultivation techniques. These systems include multi-species microbial communities formed during production of fermented food [11, 12], microbial communities in acid mine drainages and other polluted habitats [13], the commensal microbiota of corals [14], as well as several other ecosystems. In this review, we focus primarily on studies of microbe-microbe interactions in host-associated microbial communities and in relation to engineering of mixed-species microbial cell factories. We use these two examples to broadly illustrate and discuss how knowledge of species interactions is of importance in relation to our ability to control and utilize microbial systems.

Advances in studies of pathogen-microbiota interactions.

In relation to infectious diseases, it is becoming increasingly clear that interactions between bacterial pathogens and other microbial species present at the infection site (for example, co-infecting pathogens or commensal bacteria) can influence disease phenotype or clinical outcome. One example of the importance of such pathogen-microbiota interactions is the well-established role of the intestinal commensal microbiota in relation to prevention of colonization of invading microorganisms including bacterial pathogens in a process known as colonization resistance [15]. The ability to characterize microbial community structures using 16S rRNA-based phylogenies or full metagenomic sequencing has now resulted in a much deeper understanding of the interplay between the human microbiome and bacterial pathogens in relation to infectious disease development. For example, studies of the microbial communities in certain chronic infections such as cystic fibrosis (CF) have revealed clear correlations between loss of community diversity and disease progression [16-18]. CF patients are predisposed to airway infections from a number of bacterial opportunistic pathogens, among which *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae*, and *Burkholderia cepacia* complex (BCC) have been directly associated with the CF lung disease [19-21]. However, recent studies based on culture-independent methods have demonstrated the presence of many additional bacterial species previously undetected by culture and have revealed a greater microbial diversity in the CF airways than previously recognized [20]. The CF airways clearly represent a complex and diverse polymicrobial ecosystem, and as the disease symptoms become more severe, the CF lung microbiota becomes dominated by the primary pathogen (which most often is the opportunistic

pathogen *Pseudomonas aeruginosa*) [16-18]. These results are suggestive of a wider role of the respiratory microbiota, and highlight the importance of interactions between the primary pathogen and the microbiota in relation to disease progression.

There are several recent and parallel examples of interactions between the commensal microbiota and possible pathogens which are responsible for limiting colonization and infections by bacterial pathogens such as *Staphylococcus aureus* in the nasal cavity [22], and enteropathogenic *Escherichia coli* [23], and *Vibrio cholera* [24] in the gut. Despite these exciting observations, we are still far from being able to efficiently harness the protective capability of the commensal microbiota against pathogens. Nevertheless, these and related findings clearly point toward chemical and/or biological interference with microbial interaction networks within diseased hosts as alternative treatment strategies against pathogens.

The findings mentioned above highlight the importance of research aimed at systematic mapping of interspecies interactions in relation to different types of bacterial infections, in combination with identification and molecular characterization of these interactions. In other words, it is now critical to move beyond correlative research and studies focused on generating microbiome ‘parts’ lists, and instead begin to focus on causality and function at the molecular level. Indeed, a few pioneering studies have recently illustrated these points very clearly, and there are now clear examples of identified microbe-microbe interactions mediated by bacterial metabolites and gene products that function either to limit pathogen colonization [22-25] or potentiate pathogen expansion or virulence [26-28]. Although it is obviously challenging to identify and characterize microbial interspecies interactions in infected hosts, interdisciplinary approaches that combines classical microbiological *in vitro* cultivation techniques with advancing technologies such as three-dimensional (3D) printing [29], imaging mass spectrometry [28, 30], and development of realistic and controllable *in vitro* model systems [31], now makes it possible to begin to systematically tease apart the interactions among cultivated key community members, and to determine how these interactions modify pathogen behaviors.

Engineering synthetic multispecies communities for bioproduction purposes.

In Nature, microbes form interacting mixed-species communities to accomplish complex chemical conversions through division of labor among the individual organisms. We have successfully harnessed the power of such *natural microbial communities* in food and other

industries for decades [32, 33], and this has logically led to the emerging concept of community-based cell factories in which *synthetic microbial communities* are rationally designed and engineered to produce valuable chemicals. Recent studies have indeed demonstrated the potential value of such engineered mixed-species communities as production platforms. In one recent example, a synthetic mixed-species community of *Escherichia coli* and *Saccharomyces cerevisiae* was engineered to produce complex pharmaceutical molecules including precursors of the anti-cancer drug paclitaxel [34]. By engineering the two organisms to host specific portions of the biosynthetic pathways, it was possible to construct a co-culture system in which an intermediate metabolite was first produced by *E. coli* and then further functionalized by *S. cerevisiae* to give the final product. This study is the first demonstration of segregation of long and complex biosynthetic pathways into separate organisms each carrying portions of the pathway, which not only enable parallel optimization of the independent pathway modules, but also makes it possible to use the best match between particular pathway modules and specific hosts. In another recent study, a fungal-bacterial community was engineered to convert lignocellulosic biomass into biofuels [35]. Here, the community contained the fungus *Trichoderma reesei*, which can hydrolyse lignocellulosic biomass into soluble saccharides, and the bacterium *E. coli* which can metabolize these saccharides into isopropanol. In this example, one species only provided the carbon source for the second species, which in turn was able to produce the final product on its own. It is clear from these and other studies that successful engineering of community-based microbial cell factories rely greatly on our molecular understanding of microbe-microbe interactions, and how these influence community assembly, stability and activity.

Controlling the stability of community-based cell factories.

Unlike their natural counterparts, synthetic communities are often unstable. For example, different growth rates among the constituent organisms and secretion of toxic metabolites during growth can influence the stability of the community and will often lead to single-species domination or extinction of the community [36]. This general instability of synthetic communities limits their translation into real-world applications in industrial biotechnology, and achieving long-term maintenance of synthetic communities is a significant challenge that must be solved.

Although we still have incomplete understanding of the multiple competitive and cooperative interactions that control microbial community assembly and activity, many

different strategies have been successfully employed to increase the stability of synthetic communities. In the first example described above, Zhou et al [34] used knowledge of the metabolic capacities of the constituent organisms to construct a specific environment that favoured community stability: *E. coli* can use xylose as carbon source, but when grown on this carbon source, *E. coli* excretes acetate, which is inhibitory to its own growth. On the other hand, *S. cerevisiae* can use acetate as carbon source but not xylose. The use of a specific carbon source (in this case xylose) thus created a mutualistic interaction between the two organisms, which in turn stabilized the community.

In the other mixed-species community (containing *T. reesei* and *E.coli*) described in the previous section, Minty et al [35] took advantage of the particular co-operator/cheater relationship that existed in their engineered fungal-bacterial community, and used ecological theory to establish specific conditions (in terms of population sizes) that could stabilize this interaction.

However, community-stabilizing culture conditions – similar to the ones described in these two examples - may be difficult to design and construct for other synthetic communities. Most likely, it is reasonable to expect that alternative approaches will be required in most other situations. These alternative methods may include construction of synthetic interactions by genetic engineering of the participating species to enforce their interaction. For example, genetic construction of pairs of auxotrophs that cross-feed and support growth of each other when co-cultured has been shown to be an effective approach for improvements of community maintenance [37, 38]. Other strategies have relied on programming specific mutualistic interactions by means of synthetic intercellular signalling circuits [39-41]. However, such synthetic interactions are of course also targets of evolutionary process and the long-term stability of these genetic modifications is currently not well understood.

Form and function in microbial communities.

A fundamental principle in biology is that structure (form) and function are inseparable elements. For example, spatial separation of cells that are then subsequently linked together through controlled proximity is an organizational theme frequently observed at all levels in biology [42]. In relation to natural microbial communities, it is well established that spatial organization of the component species has significant impact on the function and activity of the systems [43-45]. Interestingly, such structure/function considerations are often not included in the design of synthetic microbial communities, or considered in relation to

human infections where the spatial and dynamic distribution of bacteria (including pathogens) and their activities within the human host has been found to be more complex than previously realized [46-48].

In relation to construction of community-based cell factories, it is certainly a possibility that alternative community-stabilizing methods should build on knowledge of structure/function relationships. Indeed, it has been shown that spatial separation and artificial positioning of cells within synthetic microbial communities improves community function and stability [36]. Recent advances in fluidics-based bacterial cultivation chambers [49], 3D printing methods [29], and other micro-patterning techniques [50] represent exciting areas in this direction that may advance our ability to efficiently design and control the spatial organization of cells within microbial communities.

Summary

As members of either infection communities within colonized hosts, or part of synthetic communities for sustainable bioproduction, both pathogenic and industrial relevant bacteria are placed in polymicrobial environments in which interactions and spatial position modulates their activity. In both areas there is a clear need to move beyond the current sequenced-based technologies often used to characterize complex microbial communities, and begin to identify and characterize the function of microbial interactions and the role of spatial organization. The examples shown here illustrate that such knowledge can provide new strategies for better control of bacterial infection and optimized utilization of community-based microbial cell factories. Finally, we emphasize that although our discussion is focused on examples of multispecies bacterial systems in relation to disease and biosynthesis, we believe these are indeed representative examples of an awakening field within microbial ecology focused on understanding species interactions in many types of polymicrobial ecosystems.

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